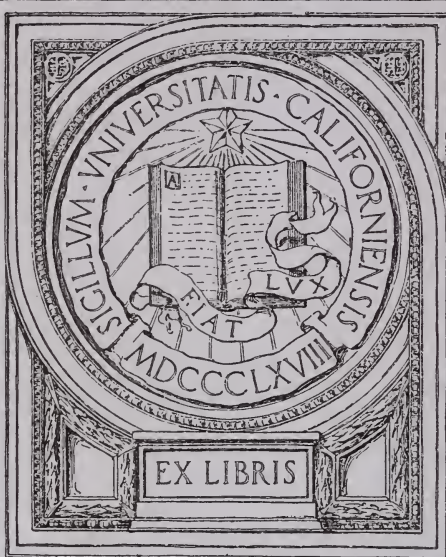




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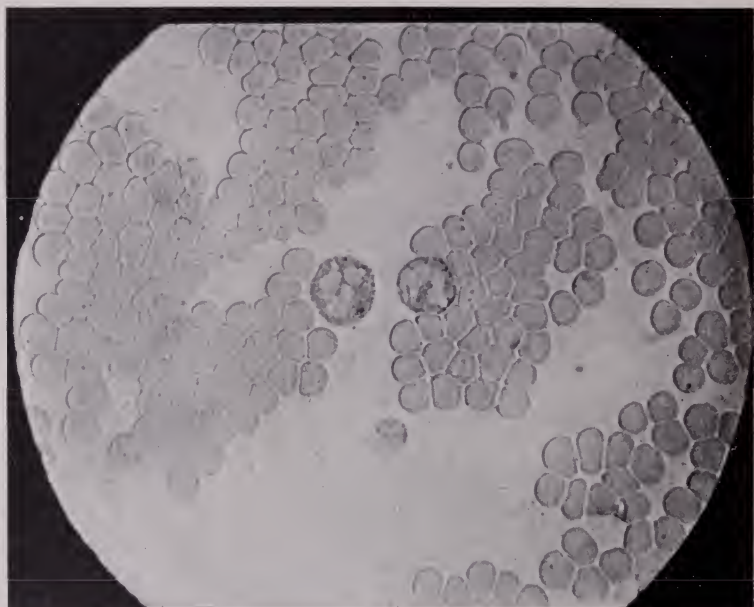


FIG. 1.—A typical field seen by the *in-vitro* method of staining. The leucocytes are staining gradually.

# INDUCED CELL-REPRODUCTION AND CANCER

THE ISOLATION OF THE CHEMICAL CAUSES OF NORMAL AND OF AUGMENTED, ASYMMETRICAL HUMAN CELL-DIVISION

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WITH 129 ILLUSTRATIONS

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SIR WILLIAM P. HARTLEY, J.P.  
OF LIVERPOOL  
AND  
JOHN HOWARD McFADDEN, Esq.  
OF PHILADELPHIA



MITOTIC FIGURE INDUCED IN A LARGE LYMPHOCYTE.

This illustration was obtained after the book had gone to press and does not appear in the list of illustrations. It is included because it so clearly demonstrates the mitosis of the lymphocyte which was unstained. The division was induced by means of bensamidine, one of the several compounds containing the amidine grouping ( $\text{N}=\text{C}-\text{N}$ ) the presence of which appears to be necessary in a substance before it can cause cell-division. This point was determined after the book had gone to press.

## PREFACE

---

THE objects of this book are to describe in detail the results obtained by a new method of experimentation with individual *living* human cells, their importance in the elucidation of the phenomena of healing, and in the causation of cancer and other growths.

The old methods of examining dead tissues and cells have been useful in the past, but I venture to think that those who undertake the study of living human cells, and especially blood-cells, by the *in-vitro* methods of staining, which will be hereafter described, will realise that they supersede all others. This method enables us to observe cells in their proper shapes, and an entirely new impression is obtained concerning the functions of their constituent elements and of the modes by which they divide and reproduce themselves. The fact that the divisions of reproduction can be induced on a microscope slide by means of the natural chemical agencies which cause their proliferation within the body has in itself opened a fresh vista of research which has not only taught us the cause of proliferation of cells in healing, but has also suggested that the cause of malignancy, which appears to be related to that normal process, is beginning to become cleared up.

The methods which I shall describe are entirely

new; some of the details have already been published in the scientific journals, but the greater part of what is herein set forth has hitherto been unknown. The new methods have revealed many interesting facts which in my opinion may be far-reaching in their influence in the advancement of pathology.

It may not be out of place if I give a brief history of the circumstances which led to the adoption of this *in-vitro* method of microscopical investigation and of the researches which have been made by means of it. There can be no doubt that accidents have on more than one occasion been responsible for valuable indications which have led to fruitful lines of work, and had it not been for some of these accidents the results attained would have been considerably less advanced than they are now. I do not think that these researches would have been started at all had it not been for the firing of a gun. In the summer of 1905, when I was a surgeon in the navy, my cabin being my laboratory, I was interested in bacteriology, and was endeavouring to grow organisms from the blood of patients. To do this I had to invent an electric incubator, it being impossible on board a battleship to use one which was heated either by gas or oil, the former not being available and the latter not allowed. There was nothing for it, therefore, but to invent an incubator which could be made on board, and I look back upon this piece of apparatus with interest. It was not so reliable as those which can now be bought, but it worked fairly well. It had an automatic thermostat, by means of which the lamp was



switched out at a given temperature, and was switched on again when the temperature fell. Sparking gave trouble, but I "blew out" the spark by a condenser. It was made by a "torpedo instructor," and was so firmly bolted on to the steel bulkhead in my cabin that apparently *nothing* would (or could) shake it down.

One afternoon, when my ship was in the Mediterranean, we had, as I thought, finished heavy gun-firing. I had placed some blood on to some nutrient agar (sloped) in culture-tubes, which were in my incubator, being kept at the blood temperature of  $37^{\circ}$  C. I was working at the cabin table with the microscope and my small stock of bacteriological apparatus. Suddenly, without any warning, a "young gentleman" fired a 12-inch gun from the after-babette on the deck above; for the captain had permitted the midshipmen to fire a "round" after the main gunnery practice was over. I extricated myself from the débris of microscope, apparatus, pictures, etc., on the deck of my cabin, for nearly everything was smashed. My incubator, firmly fixed, as I have explained, on to the bulkhead, I did not open, expecting that everything inside was shattered, and it was not until the next day that I investigated its contents. My surprise may be imagined when I found that the culture-tubes were unharmed, but that, owing to the dislocation of the automatic thermostat, the temperature inside the apparatus was standing at  $60^{\circ}$  C. On close examination of the culture-tubes, I noticed that the red cells, which had been resting on the surface of the jelly, were now diffusing as a cloud through the jelly itself. The matter was further investigated, and it

formed the subject of my paper in *The British Medical Journal* of May 5, 1906, on "The Diffusion of Red Blood-corpuscles through Solid Nutrient Agar." The reason why the diffusion had not been previously observed was that one does not usually endeavour to obtain cultures from the blood at 60° C., nor should I have done so had it not been for the zeal of the "young gentleman."

In January, 1906, before my paper was published, I was demonstrating this remarkable diffusion of red cells through the agar to my brother, Professor Ronald Ross, at Liverpool, by placing some blood under a cover-glass on a film of agar jelly spread on a slide. He was impressed by the way in which the cells became spread out between the cover-glass and the surface of the agar film, and he suggested that it would be a good means for blood-examination by the microscope, for the corpuscles became admirably arranged and spread out in such a way that each could be critically examined. Then he remarked—a remark which has led to all the researches described in this book, and to the discovery that mitotic divisions in human cells are induced by chemical agents—"I wonder what would happen if we were to mix some stain with the jelly and then place the living cells on it under a cover-glass." This suggestion was promptly put into operation, and fortunately (because it was the best which could have been chosen) the first stain experimented with happened to be polychrome methylene blue, with which I obtained results which determined me to adopt this method of examination to the exclusion of all others.

In July, 1906, I left the navy and proceeded to Egypt, having received an appointment there in the Public Health Department. Sir Horace Pinching, the Director-General, permitted me to continue the researches, and it was during the ensuing year that the phenomena of achromasia and liquefaction of the cytoplasm of leucocytes were investigated together with some of the laws concerning the diffusion of substances into cells. In January, 1907, I accidentally discovered the excitation of amoeboid movements caused by atropine, for, as will be described in the chapter relative to this phenomenon, I was in reality trying to poison the cells with the alkaloid.

In October, 1907, Sir Horace Pinching, my chief, having retired, I was treated in such a manner by Mr. W. P. Graham, the new Director-General of the Public Health Department, that I was forced to leave the Egyptian Government Service in December, 1907. Mr. Graham objected to my doing scientific work during my spare time, and also prevented my continuing the mosquito campaigns which I had started, as he apparently did not believe in them. This treatment stopped my researches for the time being, and was the cause of considerable delay in accomplishment of this work. I was enabled, however, to complete my investigations into the cause of achromasia by Dr. Marc Armond Ruffer, C. M. G., who came to my rescue and temporarily gave me an appointment in the Quarantine Department at Suakim in February, 1908. Here I was also able to devise the technics for "measuring the lives of leucocytes."

In July, 1908, I obtained the appointment of Pathologist to the Royal Southern Hospital at Liverpool, which I held for eight months, during which I was able to investigate further the laws of the diffusion of substances into living cells and to devise the technics for the determination of the "coefficient of diffusion." In the meantime I published the results of work done while I was in Egypt. This could not be done before, because Mr. Graham would not permit me to publish scientific work. Two papers appeared in *The Journal of Physiology* in September, 1908, four in *The Lancet* in January and February, 1909, and that on the "Coefficient of Diffusion" in the *Proceedings of the Royal Society* in April, 1909.

In August, 1908, I was demonstrating the excitation of amœboid movements caused by atropine to Dr. Macalister, when he suggested to me that possibly there might be some alkaloid-like excitant in the blood of cancer patients; and this important suggestion was the starting-point of the investigation of cancer by this *in-vitro* method. Dr. Macalister and I read a paper before the Royal Society of Medicine in November, 1909, on the researches which immediately followed his suggestion.

In March, 1909, Professor Harvey Gibson also suggested the important point, based on an observation made by Professor Farmer, that nuclein might have some influence on cell-division. I must acknowledge the great assistance which I have received from Professor Harvey Gibson on many occasions, as well as the loan of a well-equipped laboratory in the Hartley

Botanical Department at the University of Liverpool.

It was when experimenting with a mixture of polychrome stain, extract of hæmal gland, and atropine that I saw mitotic figures in lymphocytes for the first time in May, 1909; and the ensuing months were occupied in the investigation of the cytology of these cells and of the means whereby they might be induced to reproduce themselves. It was not until October, 1909, however, that I was able to induce divisions in polymorphonuclear leucocytes. In December, 1909, I discovered almost accidentally that extracts of dead tissues, if they were allowed to decompose by the action of putrefactive bacteria, would, by themselves, induce the division and multiplication of lymphocytes, and this was followed by the investigation of the action of "globin" in January, 1910. In February, 1910, while investigating the epithelial cells present in some vaginal secretion, it occurred to me to try to induce divisions in them, and this experiment has been successful in the case of one or two cells.

In April and May, 1910, when working with my assistant, Dr. Cropper, I saw divisions induced by kreatin and xanthin, the "extractives" contained in the remains of dead tissues; and we then also investigated the augmenting action on cell-division of the alkaloids choline, cadaverine, etc., produced by the decomposition of putrefaction. These points led me to elaborate the theory regarding the cause of cancer which is described in the latter part of this book.

The dates of the treatment of the two cases of cancer are given in the description of them which



has been written by Dr. Macalister. The crucial experiment to try to determine whether my theory regarding the cause of cancer was correct or not was made in the first week of August, 1910. The treatment of all the cases has been carried out under the immediate supervision of Dr. Macalister.

In much of the latter portion of the experimental work I have derived assistance from Dr. Cropper. He has accomplished nearly all the investigations concerned in counting the number of granules contained in eosinophile leucocytes, and has given most valuable assistance in the isolation of the active "auxetics" from the remains of dead tissues, and in the investigation of the inhibitory action of blood-serum, some of which he did entirely himself at my suggestion.

The Research Department at the Royal Southern Hospital at Liverpool was started by Dr. Macalister in April, 1909, in order that this work might continue; Sir William Hartley, J. P., generously supplied funds to last for one year in the first instance. In November, 1909, he extended his support for a period of three years, and he also supplied me with the photomicrographic camera which I had invented. Some of the expenses attached to these researches, however, have also been defrayed by Mrs. George Holt, Mr. and the Misses Paton, and some of their friends. I would like to take this opportunity of recording my personal gratitude to Sir William Hartley and these ladies and gentlemen, without whose assistance these researches could not have been accomplished.

I wish also to record the manner in which I was

enabled to obtain the assistance of Dr. Cropper. In October, 1909, I happened to be discussing with Mr. Sharples, an energetic supporter of these researches, the difficulty of my finding time to undertake the investigation of some of the by-issues revealed by the new method—issues which might prove to be of importance. Our conversation was overheard by a gentleman, who I afterwards ascertained was Mr. J. H. McFadden, of Philadelphia, whose acquaintance I had only just made, and to whom I was practically a stranger. Mr. McFadden immediately became interested, and placed a large sum of money at my disposal in order that I might obtain other assistance to further these researches for a period of two years. In March, 1910, Mr. McFadden further instructed me to the effect that if I conscientiously thought that further funds could usefully be spent in the advancement of these researches I was to incur that expenditure. In fact, he has not only supplied me with the assistance of Dr. Cropper, but he has also been the means of equipping a laboratory for him, kindly lent by the Liverpool School of Tropical Medicine, but also in defraying the serious expenditure connected with the manufacture of the substance we call “globin” from crystalline hæmoglobin. Mr. McFadden has also enabled me to take the large number of photomicrographs which record the phenomena seen under the microscope; and, lastly, he has borne the entire cost of the publication of this volume and the reproduction of the photomicrographs which illustrate it.

I fear that I shall never be able to thank Mr.

McFadden sufficiently for his great generosity, which I appreciate very greatly. It was extended to me at a time when I was practically a total stranger to him, together with the intimation that even if these researches did not result in the advancement of knowledge regarding cancer he would not consider that his assistance had been misplaced or wasted.

In conducting prolonged researches of this nature it is most gratifying to realise that one has such a staunch supporter; and there can be no question that if the results obtained lead to practical benefit, this will be largely owing to Sir William Hartley, who enabled the researches to be started, and to Mr. McFadden, who enabled them to be brought so rapidly to the point which has now been reached.

When this Research Department of the Royal Southern Hospital was started, a Committee was formed. It numbers amongst its members Professors Sherrington, Herdman, Ronald Ross, Reynolds Green, and Harvey Gibson, to all of whom I have frequently appealed for advice on technical points; and when any information has been required concerning the surgical aspects of the healing process, or of cancer, I have consulted Mr. Robert Jones, who is now Chairman of the Committee, and also Dr. Alexander. I wish to thank all these gentlemen most sincerely for their kindness. I have also frequently received materials from Mr. Jeans and Mr. Bickersteth, of the Royal Infirmary, and many other members of the medical profession in Liverpool have supplied specimens. The beautiful sections of the growth with which the crucial

experiments were made were kindly cut for me by Dr. Moore Alexander. Professor Benjamin Moore advised us in our researches to isolate the active auxetics from the extracts of dead tissues, and Dr. H. E. Roaf very kindly supplied us with the crystalline hæmoglobin from which globin was obtained for the first experiments with that substance.

I cannot close this Preface without signifying my thanks to Dr. Macalister. He grasped that this *in-vitro* method would bring fruitful results, and it was he who instituted this research into the cause of cancer. Dr. Macalister's advice and constant encouragement, apart from the actual experimental work which he has done and the clinical observations which he has made, have been invaluable.





# CONTENTS

---

## CHAPTER I

	PAGE
THE SCOPE OF THE NEW METHOD . . . . .	1

## CHAPTER II

THE GENERAL PRINCIPLES OF THE METHOD—THE APPARATUS REQUIRED—THE SPECIAL PHOTOMICROGRAPHIC APPARATUS—THE REVOLVING APPARATUS . . . . .	15
---	----

## CHAPTER III

THE PREPARATION OF THE JELLY-FILM . . . . .	36
---	----

## CHAPTER IV

CELLULAR STAINING, DEATH, AND ACHROMASIA . . . . .	41
--	----

## CHAPTER V

THE DIFFUSION OF SUBSTANCES INTO LIVING CELLS—THE “COEFFICIENT OF DIFFUSION” . . . . .	61
--	----

## CHAPTER VI

THE PRACTICAL DETERMINATION OF THE “COEFFICIENT OF DIFFUSION” OF CELLS, AND ITS APPLICATION TO THIS “IN-VITRO” Method of Research . . . . .	81
---	----

## CHAPTER VII

PAGE

DIFFUSION OF SUBSTANCES INTO CELLS TO EXCESS—DIFFUSION-VACUOLES OR "RED SPOTS"—THE PROOF THAT THE BLOOD-PLATELET IS A LIVING CELL . . . . .	103
---	-----

## CHAPTER VIII

THE EXCITATION OF AMŒBOID MOVEMENTS IN WHITE BLOOD-CORPUSCLES CAUSED BY ALKALOIDS . . . . .	130
---	-----

## CHAPTER IX

THE ADOPTION OF THE "IN-VITRO" METHOD FOR CANCER RESEARCH—THE EXCITATION OF LEUCOCYTES CAUSED BY CANCER PLASMA—FACTS KNOWN ABOUT CANCER—THE AGE-INCIDENCE; VITALITY; DEATH; METASTASIS; CHRONIC IRRITATION—THE POSSIBLE CAUSES OF CELL-PROLIFERATION DISCUSSED . . . . .	157
--	-----

## CHAPTER X

EXPERIMENTS WITH NUCLEIN—THE LOWERING OF THE COEFFICIENT OF DIFFUSION CAUSED BY EXTRACTS OF DEAD HÆMAL GLAND—DIVISIONS INDUCED IN LYMPHOCYTES FOR THE FIRST TIME—REVELATIONS CONCERNING THESE DIVISIONS—THE ROLES PLAYED BY THE ALTMANN'S GRANULES, NUCLEI, AND NUCLEOLI IN THE CELL-DIVISION	172
---	-----

## CHAPTER XI

THE DIVISION OF LYMPHOCYTES INDUCED BY THE ANILINE DYE—THE AUGMENTING ACTION OF ATROPINE AND EXTRACT OF HÆMAL GLAND—"AUXETICS"—THE CYCLE OF CELL-DIVISION—THE POSSIBILITIES OF THE INDUCED CELL-DIVISION BEING DUE TO "DEATH-STRUGGLES"—ASYMMETRICAL AND REDUCED DIVISIONS . . . . .	225
--	-----

## CHAPTER XII

PAGE

THE "EXPERIMENTAL TEN MINUTES"—DIVISION INDUCED IN THE SO-CALLED POLYNUCLEAR LEUCOCYTES—METHOD FOR COUNTING THE NUMBER OF GRANULES IN EOSINO- PHILE LEUCOCYTES, AND THE REDUCTION OF THIS NUM- BER IN THE CELLS OF CANCER PATIENTS . . . . .	249
--	-----

## CHAPTER XIII

THE AUXETIC ACTION OF CANCER-SERUM—THE INDUCED DIVISIONS OF GRANULAR RED CELLS—THE AUXETIC ACTION OF "THE REMAINS OF DEAD TISSUES," AND ITS AUGMENTATION BY ATROPINE AND THE PRODUCTS OF PUTREFACTION—THE ISOLATION OF THE AUXETICS KREATIN AND XANTHIN—DISCOVERY OF CAUSES OF THE CELL-PROLIFERATION OF HEALING . . . . .	292
--	-----

## CHAPTER XIV

THE AUXETIC ACTION OF GLOBIN . . . . .	322
--	-----

## CHAPTER XV

THE PROOF THAT THE REMAINS OF DEAD TISSUES AND GLO- BIN CONTAIN THE CAUSES OF THE CELL-PROLIFERATION OF HEALING, AND OTHER CELL-REPRODUCTION—EXPERI- MENTATION "IN VIVO" CONFIRMS "IN-VITRO" OBSERVA- TIONS—THE CAUSE OF BENIGN TUMOURS . . . . .	333
---	-----

## CHAPTER XVI

THE AUGMENTED DIVISIONS INDUCED BY PUTREFACTION OF THE EXTRACTS IS DUE TO THE ALKALOIDS OF PUTRE- FACTION—A THEORY THAT CARCINOMA AND LYMPHA- DENOMA MAY BE CAUSED BY THE COMBINATION OF THE AUXETICS OF CELL-PROLIFERATION AND CHOLINE AND CADAVERINE—AN EXPLANATION OF THE AGE-INCIDENCE, METASTASIS, AND THE OTHER FACTS KNOWN CONCERNING CANCER—THE NECESSITY FOR A CRUCIAL EXPERIMENT TO PROVE THE THEORY . . . . .	348
--	-----

## CHAPTER XVII

PAGE

THE INHIBITORY ACTION OF BLOOD-SERUM IN PREVENTING THE ACTION OF AUXETICS IN CAUSING CELL-DIVISION— MEASUREMENT OF THIS ACTION—THE TREATMENT OF SOME CASES OF CANCER BY DEFIBRINATED BLOOD— DESCRIPTION OF THE CASES—THE TREATMENT OF A MALIGNANT ULCER BY MEANS OF GLOBIN—THE CRUCIAL EXPERIMENT—CONCLUSION . . . . .	374
--	-----

## APPENDIX I

TABLES DESCRIBING THE ENUMERATION OF THE NUMBER OF GRANULES CONTAINED IN EOSINOPHILE LEUCOCYTES	401
--	-----

## APPENDIX II

METHOD FOR ESTIMATING THE NUMBER OF LIVING AND DEAD LEUCOCYTES CONTAINED IN A GIVEN SAMPLE OF BLOOD, AND MEASURING THE LIVES OF LEUCOCYTES . . . . .	406
--	-----

## APPENDIX III

A "HANGING-DROP" PREPARATION WITH THE JELLY METHOD	419
--	-----

## APPENDIX IV

A CONTRIBUTION TO THE "THEORY OF IMMUNITY". . . . .	420
INDEX . . . . .	421

## LIST OF ILLUSTRATIONS

ALL the photomicrographs which illustrate this book were taken with the apparatus described in Chapter II. The objective used for those taken with the higher magnification was a 2-mm. apochromatic lens (Zeiss) N. A. 1.30. The objectives employed for taking photographs of a lower magnification were Zeiss D and Zeiss A. The eye-piece used in all of them was a "high-power projection eye-piece" (Watson).

In the actual preparations, as observed through the microscope, a stereoscopic view of the dividing cells can be obtained, which facilitates the demonstration of the different phases. Unfortunately, this stereoscopic effect cannot be seen in the prints, although an examination of them with a hand magnifying-glass will remedy the deficiency to some extent.

The photographs have all been produced without any alteration of the original negatives.

FIG.	PAGE.
1. A typical field seen by the <i>in-vitro</i> method of staining. The leucocytes are staining gradually . . . . . Frontispiece.	
2. The photomicrographic apparatus. The microscope is ready to be used for direct observation. The gas-burner can just be seen at the lower end of the wooden plank. (N. B.—The sheets of white paper have been placed in this position in this and the next photograph in order to "show up" the apparatus.) . . . . .	23
3. The apparatus ready for photography. The mirror is swung aside, and the eye-piece attached to the camera is inserted into the microscope . . . . .	25
4. The photomicrographic apparatus. Showing positions of water-cooling tank and Nernst burner. The microscope mirror is in position for direct observation . . . . .	29
5. The photomicrographic apparatus. The microscope mirror is swung aside for photography . . . . .	31
6. The granules of the leucocyte are gradually becoming stained. The red cells are unstained. Low power . . . . .	45
7. The leucocyte's granules are stained. Its nucleus is unstained. The pseudopodia are extruded in response to atropine, which is diffusing into the cell as well as the stain . . . . .	45

FIG.	PAGE.
8. The same field as 7. The leucocyte is retracting its pseudopodia.	47
9. The same field as 7 and 8. The retraction of pseudopodia is nearly complete. The lobes of the nucleus of the leucocyte are turning a faint blue colour . . . . .	47
10. A leucocyte excited by atropine. Its granules are deeply stained, and its nucleus is also beginning to stain a blue colour. Low power . . . . .	49
11. A leucocyte which has just been killed by the staining of its nucleus. Its granules are also deeply stained . . . . .	49
12. The leucocyte has just died owing to the staining of its nucleus. The cell-wall is beginning to bulge because the cytoplasm is liquefying . . . . .	53
13. The onset of achromasia. The same field as 12. The stain is beginning to fade from the nucleus. The bulging of the cell-wall has become general . . . . .	53
14. Achromasia. The same field as 13. The stain has gone from the nucleus, although the granules are still stained. Note that the red cell is disappearing. . . . .	57
15. Achromasia. The same field as 14. Many of the cell-granules have lost their stain. The cell-wall is nearly invisible. The red cell has disappeared . . . . .	57
16. A stained leucocyte. The ordinary vacuoles (colourless patches amongst the cell granules) are well shown. The cell has just died . . . . .	105
17. Diffusion-vacuoles in a leucocyte . . . . .	105
18. A dead leucocyte in which diffusion-vacuoles are beginning to appear . . . . .	109
19. A diffusion-vacuole in a lymphocyte. Low power . . . . .	109
20. A diffusion-vacuole in a granular red cell . . . . .	115
21. A clump of normal blood-platelets. They are resting on a jelly which will just stain their granules . . . . .	115
22. Diffusion-vacuoles in blood-platelets. The cells are resting on the same jelly-film as those in 21, but they had been subjected to the action of morphine hydrochloride . . . . .	119
23. Diffusion-vacuoles in blood-platelets. The jelly-film had the same index of diffusion as that employed in 21 . . . . .	119
24. A specimen of blood which had been mixed with morphia solution. Note the extreme vacuolation of the leucocyte. A blood-platelet is also vacuolated. The same jelly as in 21 . . . . .	121
25. Patches resembling archoplasm induced in a leucocyte by subjecting the blood to an extract of a dead tissue. The jelly-film on which the cells are resting is similar to that employed in 21.	121
26. An extruded pseudopodium becoming detached from a leucocyte which is excited by atropine. No stain . . . . .	125
27. Amœboid movements excited in a blood-platelet by the action of atropine . . . . .	125

FIG.	PAGE.
28. Amœboid movements excited in a leucocyte by the action of atropine. Low power . . . . .	135
29. Exaggerated amœboid movements in leucocytes which have their granules stained. The movements were excited by atropine sulphate . . . . .	135
30. Excited leucocytes extruding their pseudopodia between red cells.	137
31. Excitation of amœboid movements in a lymphocyte by the action of atropine. No stain . . . . .	137
32. Excitation of amœboid movements in a lymphocyte which has its granules stained . . . . .	143
33. Extreme excitation of amœboid movements in a lymphocyte. No stain . . . . .	143
34. Excitation of two leucocytes by the action of choline. Low power. No stain . . . . .	151
35. Excitation of a lymphocyte by the action of choline. No stain .	151
36. Excitation of amœboid movements in a leucocyte by the action of cadaverine. No stain . . . . .	153
37. A leucocyte excited by morphine. The cell's granules are stained.	153
38. Leucocytes excited by pyridine. No stain . . . . .	173
39. A lymphocyte which has absorbed stain and atropine discarding its granules (flagellation). . . . .	173
40. A resting lymphocyte. Note the deeply stained masses of granules in the cytoplasm, which is bulged out in places. The large transparent nucleus and the stained ring-shaped nucleolus can also be seen . . . . .	189
41. A resting lymphocyte. The Altmann's granules in the cytoplasm are stained . . . . .	189
42. A resting lymphocyte. The cytoplasm, the granules, the nucleus, and the nucleolus can be distinguished . . . . .	191
43. The earliest stage of mitosis. The nucleolus has divided into two rings. . . . .	191
44. Early mitosis in a lymphocyte. Looking down through the spindle (polar aspect). The nucleolus has divided into two centrosomes, each of which is ring-shaped. The spindle is surrounded by a belt of chromatin granules . . . . .	193
45. Mitosis in a lymphocyte. Profile aspect. The two ring-shaped centrosomes can just be seen towards the poles. The granules are becoming formed into chromosomes . . . . .	193
46. Foreshortened appearance of a mitotic figure in a lymphocyte. The position of one nucleolus-centrosome at the pole of the figure is well shown . . . . .	195
47. Profile aspect of mitosis in a lymphocyte. The relative positions of the centrosomes and chromosomes can be seen . . . . .	195
48. Profile aspect of mitosis. The belt of chromatin is formed round the waist of the cell . . . . .	197



FIG.	PAGE.
49. One resting and one dividing lymphocyte. In the latter the chromosomes are beginning to divide. The centrosomes appear as dots of chromatin . . . . .	197
50. Polar aspect. The belt of chromatin granules is dividing into chromosomes . . . . .	199
51. Polar aspect. The chromosomes are becoming semicircular . . .	199
52. Polar aspect. An "aster" stage of mitosis in a lymphocyte . . .	201
53. Polar aspect. Some of the chromosomes are semicircular-shaped; some are dots of chromatin . . . . .	201
54. Polar aspect. One centrosome can be seen at the pole of the "aster" figure . . . . .	203
55. Polar aspect. Sixteen chromosomes could be counted in this cell .	203
56. Profile aspect. of mitosis in a lymphocyte. . . . .	205
57. Profile aspect of mitosis in a lymphocyte . . . . .	205
58. Profile aspect. The chromosomes can be seen at the waist of the spindle . . . . .	207
59. Profile aspect. A figure frequently seen . . . . .	207
60. Profile aspect of mitosis . . . . .	209
61. Oblique aspect of mitosis in a lymphocyte . . . . .	209
62. Polar aspect of mitosis in a large lymphocyte from a patient suffering from carcinoma. There are sixteen chromosomes . . .	211
63. Polar aspect. The chromosomes were V-shaped with their apices inward to be attached to the nucleus-spindle, which can dimly be made out . . . . .	211
64. Polar aspect of mitosis in a large lymphocyte from a cancer patient. The chromosomes are dividing . . . . .	213
65. Profile aspect of mitosis . . . . .	213
66. Profile aspect. The figure is fully formed. One nucleolus-centrosome is ring-shaped; the other is a dot of chromatin . . . . .	215
67. Profile aspect. The sixteen chromosomes could be counted . . .	215
68. The cell has become constricted in its centre . . . . .	217
69. Profile aspect. Complete division is about to occur. The chromosomes are being reconverted into granules, but the mitotic figure is not quite finished at the dividing-point . . . . .	217
70. Profile aspect. The spindle and chromosomes have divided, but the cell-wall has not yet separated . . . . .	219
71. Completion of mitosis in a lymphocyte . . . . .	219
72. Asymmetrical mitosis in a lymphocyte induced by azur stain augmented by atropine . . . . .	233
73. Asymmetrical mitosis induced by azur stain augmented by atropine . . . . .	233
74. An early stage of delayed mitosis induced by a jelly with a low index of diffusion. The number of chromosomes is more than sixteen . . . . .	241
75. Thirty-two chromosomes could be counted in this cell. Early mitosis delayed . . . . .	241

FIG.	PAGE.
76. A resting polymorphonuclear leucocyte. Its granules are stained but not its nucleus. The cell was alive . . . . .	253
77. A basophile leucocyte in the act of cell-division. The granules of the cell are in the centre. The lobes of the nucleus are at the poles of the cell which is dividing into three . . . . .	253
78. An eosinophile leucocyte in the earliest stage of division. The granules were arranged in lines radiating outwards from the centre of the cell. The lobes of the nucleus were at the poles. . . . .	259
79. Early stage of division of a neutrophile leucocyte . . . . .	259
80. A dividing leucocyte . . . . .	261
81. A dividing leucocyte . . . . .	261
81A. Division of a leucocyte. The linear arrangement of the granules could be well seen . . . . .	263
82. A dividing leucocyte . . . . .	263
83. A dividing leucocyte . . . . .	265
84. A dividing leucocyte . . . . .	265
85. A dividing leucocyte . . . . .	267
86. A dividing leucocyte . . . . .	267
87. An eosinophile leucocyte with its granules stained . . . . .	275
88. A field containing a neutrophile, an eosinophile, and a basophile leucocyte. The upper cell is the neutrophile and the lower one the basophile cell. All the cells are ruptured, but their granules are stained . . . . .	279
89. A basophile leucocyte whose stained granules have been turned black by heat . . . . .	279
90. One of the negatives of a ruptured eosinophile leucocyte (negative No. 52) . . . . .	285
91. One of the negatives of a ruptured eosinophile leucocyte (negative No. 54) . . . . .	285
92. Counting the granules. The image of the ruptured cell depicted on negative No. 52 is projected on to a sheet of white paper pinned on to a screen . . . . .	287
93. Counting the granules of negative No. 54 . . . . .	287
94. A dividing red cell from a cancer patient . . . . .	295
95. A dividing red cell from a cancer patient. The granules seem to be arranged in an indefinite figure . . . . .	295
96. Very early stage of mitosis in a lymphocyte induced by decomposed extract of suprarenal gland. No stain . . . . .	301
97. Mitosis of a lymphocyte induced by decomposed suprarenal extract. No stain. . . . .	301
98. Mitosis induced in a lymphocyte by decomposed extract. No stain. . . . .	303
99. Asymmetrical division induced by decomposed extract. No stain or atropine is present . . . . .	303
100. Mitosis induced by fresh extract of suprarenal gland. No stain or augmentor present . . . . .	307

FIG.	PAGE
101. Mitosis induced by fresh suprarenal extract. No stain is present.	307
102. A dividing polymorphonuclear leucocyte induced by suprarenal extract alone. No stain . . . . .	311
103. Mitosis induced in a lymphocyte by suprarenal extract which had purposely been allowed to become putrid. No stain . . . . .	311
104. Mitosis induced in a lymphocyte by suprarenal extract which had purposely been allowed to become putrid. No stain . . . . .	313
105. Asymmetrical mitosis induced by suprarenal extract augmented by atropine. No stain . . . . .	313
106. Mitosis induced in a lymphocyte by kreatin. No stain or extract.	317
107. Division in a leucocyte induced by kreatin. No stain or extract.	317
108. Mitosis in a lymphocyte induced by globin augmented by atropine. No stain, extract, or kreatin . . . . .	327
109. Asymmetrical mitosis induced by globin augmented by atropine. No stain, extract, or kreatin . . . . .	327
110. Mitosis induced in a lymphocyte by means of decomposed globin solution. No stain, extract, kreatin, atropine. . . . .	329
111. To show the way in which globin is "dotted" over the surface of an ulcer . . . . .	343
112. To show the scab formed by the application of globin to an ulcer.	343
113. Mitosis induced by a mixture of kreatin and choline. No stain, extract, or atropine . . . . .	353
114. Asymmetrical mitosis induced in a lymphocyte by a mixture of suprarenal extract and globin, augmented by choline. No stain or atropine . . . . .	353
115. Mitosis in a lymphocyte induced by globin and choline. No stain or other auxetic . . . . .	355
116. Mitosis induced in a lymphocyte by suprarenal extract and choline. No stain or other auxetic . . . . .	355
117. Mitosis induced in an epithelial cell by a mixture of stain and extract. . . . .	357
118. Early mitosis in an epithelial cell from the vagina induced by stain and extract . . . . .	357
119. Section from the case of scirrhus of the breast. Low power . . .	385
120. The same as 119. High power . . . . .	385
121. To show the way in which globin was "dotted" on to a portion of the malignant ulcer . . . . .	387
122. Section of a portion of the ulcer after treatment. Low power . .	391
123. The same as 122. High power . . . . .	391
124. Section of the treated portion of the ulcer after the application of globin augmented by choline, showing reinfiltration. Low power . . . . .	393
125. The same as 124. High power . . . . .	393

# Induced Cell-Reproduction and Cancer

## CHAPTER I

### THE SCOPE OF THE NEW METHOD

THE study of the individual living human cell and of the effects of chemical reagents upon it marks what may almost be regarded as a new scientific departure. Although much has been written concerning the passage of substances into cells, mainly the outcome of experiments not made actually upon the individual cells themselves, and certainly not while they were alive, little practical work has been done with reference to the behaviour of individual cells while substances are being made to pass into them. This has been owing to the lack of satisfactory methods, and because the laws which govern the diffusion of substances into the individual living cells have not been recognised. These laws are of the greatest importance, and must be thoroughly understood if *in-vitro* experimentation is to prove serviceable or successful, and later on a section will be devoted to this subject. In the meantime the elemental fact must be simply stated that living cells are examined by placing them, under a cover-glass, on to the surface of a film of jelly, which may contain dissolved in it any substance we may wish

to experiment with, and which has, while in a molten condition, been poured on to a microscope slide and allowed to set there. The jelly may, for instance, contain an aniline dye; and by watching the way in which the living cells absorb the stain from the jelly, and by experimentation with it, many of the laws of the diffusion of substances into living cells have been ascertained; and by the application of these laws we can now add other substances to the jelly and make them also diffuse into the living cells, and watch the results by means of the microscope. The cells are pressed into the jelly by the cover-glass, and therefore they can absorb only what is in the jelly (there is nothing else for them to take), provided that the conditions have been correctly arranged for the passage of the substances from the jelly into the cells. It is essential to note that one class of cells differs from another with reference to the rate at which they absorb materials from the media in which they are placed, so that the composition of any given jelly must be correctly arranged for experimentation with any particular class of cell with which it may be desired to work.

The word "cell" in this book refers to the living cell unless otherwise specified. Cells must always be freshly removed from the body when they are placed on the jelly. It occasionally happens that the cells may have just died or be dying when they are examined, as when mitotic divisions are being induced by azur stain, as will presently be described; but, generally speaking, after the cells in a specimen are dead the specimen is thrown away. It is obvious



that "specimens" of living cells cannot be kept. All attempts to "fix" the jelly films (on which the cells are resting) at the end of the experiments have so far failed, so it is impossible to retain the specimens for future examination or for purposes of collection; and consequently when dead, or when finished with, specimens have to be discarded. This circumstance has led me, at the suggestion of Professor Sherrington, to devise a rapid method of recording the actual experimental facts observed by means of photomicrography; and although the photographs, many of them taken with the highest powers of the microscope, are not comparable by any means to what is seen with the eye, we at least have the satisfaction of knowing that a truthful image is recorded which cannot be influenced in the way that drawings, however carefully made, are apt to be. The photomicrograph is therefore the best substitute for microscope "specimens" which we have to offer.

In the past very little has been learned from the study of individual living cells either in physiology or in pathology. Presumably this has been due to the fact that it has been difficult to stain cells satisfactorily when they are alive; for, since the discovery of the aniline dyes, stains have been used in nearly all microscopical work. It is true that a good deal of work has been done in the way of attempting to stain unfixed cells by mixing them with solutions of methylene blue and neutral red; but the results have not been very satisfactory, and no doubt the advances made in the study of dead cells by means of differential staining

with dyes dissolved in alcohol have done something to retard *in-vitro* methods of investigation, because dyes dissolved in alcohol cannot, of course, be used to stain living cells. As a matter of fact, with this new "jelly" method it is simpler to stain certain living cells than it is to stain dead ones by the old methods, and better pictures are obtained although less skill is required. No matter how rapidly a cell or tissue is killed, the fact remains that it is dead, and the means usually taken to prepare it for examination by placing it in preservative solutions or in others necessary for fixing and staining it—not to speak of the processes of embedding and freezing and the subsequent cutting with razors and so forth—can only add to the fallacious results of its examination. So far as blood-cells are concerned, the study of their morphology and cystology has hitherto been almost entirely based on the examination of dead specimens, with the result that some erroneous impressions, both as to form and function, have become generally accepted. For instance, let an experienced worker with the older methods look for a "hyaline leucocyte" with the new one, and he will marvel at his credulity. The hyaline leucocyte is a dead lymphocyte which has become achromatic. By the new method we see cells stained while they are alive, and admirably spread out on the jellies, so that they can readily be examined by the highest powers. One can now cause any soluble substance to diffuse into them at any rate one pleases, and with the help of this knowledge one can, by specific chemical agents, cause leucocytes and other cells to divide on



the microscope slide. By this study of vital activity new lessons have been learned concerning the real functions of the morphological elements of the cells. For instance, owing to the fact that the older methods merely showed pictures of dead cells and the arrangement of their component parts after they are dead, controversies have arisen regarding the functions of the cellular elements. Unfortunately, theories regarding these functions have sometimes become accepted as facts. The "lobes of the nuclei" of leucocytes are generally recognized as being analogous to the nuclei of other cells, in spite of the fact that the act of cell-division has never been seen in leucocytes.<sup>1</sup> The very designation of the cells—"polymorphonuclear"—is even based on this theory; but in reality the "lobes of the nuclei" are the centrosomes. We hear it said even now that the blood-platelet is a precipitate, although a single glance at a specimen *in vitro*, especially if an alkaloid is present in the jelly, demonstrates beyond denial that a blood-platelet is a living creature and a highly amoeboid cell.

The new method reveals new points in every direction which are difficult to reconcile with the old theories based upon the examination of dead specimens, some of them so firmly rooted that people may be slow to discard them.

Infinite interest and variety awaits the investigator of cells by this new method. He is dealing with living

<sup>1</sup> Throughout this book the word "leucocyte" refers to the polymorphonuclear cell; the mononuclear cell from the peripheral circulation is called a lymphocyte.

creatures which are amenable and can be excited or made to divide almost at will. It is remarkable to think that one can order samples of one's own or some other person's white blood-corpuscles to reproduce themselves at a given time, and that if they are properly treated they will do so with obedient regularity. Instead of the diagrammatic representations of karyokinesis, from which every student learns his impressions of cell-division, one is now able to appreciate mitosis in its reality and to watch it through its various phases. This is a very striking fact, but its interest grows when we consider another very important lesson derived from it, insomuch that, as will be seen later, there is strong evidence that white corpuscles will multiply only in response to a specific chemical agent. We now believe that it is essential for a leucocyte to absorb an "auxetic" (ἀνξήτικος, an exciter of reproduction) before it will make any attempt to proliferate, and we have also evidence that it is more than probable that other human cells, and possibly all of them, proliferate in response to a similar agency. It will be realized, therefore, that this method of study of the cell and of the influences of chemical agencies upon it has opened up a new field of work, not only in pathology, but in physiology also.

The proliferation of cells is the main theme of this book. By this *in-vitro* method it has not only been learnt that cells will divide in response to certain chemical agents, but that these agents exist in the remains of all dead tissues. Two of the substances which are directly responsible for cell-reproduction within the

body have been isolated in crystalline form: they are the extractives, kreatin and xanthin, and individual cells divide in response to them according to the amount of each substance absorbed by the cell.

It will be shown that cell-proliferation depends upon cell-death, and this affords an explanation of the cause and origin of benign tumours. "Development" is a basis of physiology; and since the multiplication of human cells is due to chemical agents, as is shown by this method, one cannot but suppose that the facts learnt may lead to the explanation of points connected with the growth of the embryo.

One of the foundations of pathology is the phenomenon of "healing," which is caused primarily by the proliferation of certain cells. The causes of this proliferation have been ascertained for the first time by this method, and the ultimate chapters of this book will describe proofs that these causes are now known. If the finger is cut, or if disease gains a footing in any part of the body, an attempt is made by the tissue-cells to proliferate and to heal the injury; but up to now no one has known why this proliferation took place or how it was caused. This mystery is now elucidated. The knowledge that cell-proliferation in the body is due to chemical excitors, of reproduction (auxetics) is, we think, the beginning of an innovation which must lead to developments of practical value.

The effect of any given substance, so long as it is soluble, can be tested on many individual human cells and the results watched. I fear that we, personally, have only been able so far to try the effects of auxetics,

alkaloids, and a few other substances; but a whole field of investigation of the actions of substances on individual cells remains to be carried out, and this is now possible by this "jelly" method of *in-vitro* staining.

The action of chemical substances on living cells is closely associated with the diffusion of substances into these cells (a subject to which a section of this book will be devoted), and this diffusion is governed by the "coefficient of diffusion" of the cells themselves, a phenomenon which has been so far entirely studied by this *in-vitro* method. Up to the present, however, we have only had time to ascertain the comparative rates of diffusion of substances into some of the classes of human cells and into a few species of bacteria. The determination of the coefficients of diffusion of all the rest of the cells of the whole animal and vegetable kingdoms remains as a "legacy" for those who will undertake the work.

Methods will be described by which the lengths of the lives of leucocytes can be measured after they have been removed from the body. By this means the comparative effects of different poisons on the cells can be tested, and the small amount of work done in this direction will be summarised. We think that there are possibilities that farther investigation of the actions of specific poisons, such as bacterial toxins, will lead to fruitful results; in fact, one of us (C. J. M.) has already shown by this method that chorea and rheumatism are less closely related than is generally supposed.<sup>1</sup>

<sup>1</sup> *British Medical Journal*, August 28, 1909.

In the last chapter experimental evidence is given to prove that blood-serum has an inhibitory action on cell-division; and it will also be seen that it is possible to measure this inhibitory action. Since the cell-proliferation of healing is caused by chemical substances contained in the soluble remains of dead tissues, and since, as will be shown, bacteria decompose these solutions, a field of research is opened for the investigation of this decomposing action by various pathogenic bacteria; for in decomposing the sources of the causes of healing they greatly modify that process, and the healing process must play an important part in immunity against disease. Further, bacteria may have an action on the substances contained in blood-serum which restrain cell-division. We fear that we have hitherto been able to do little towards the investigation of this factor in the problem of immunity, which is now mentioned for the first time.

These are only a few of the fields which have been pried into by experimentation with this new method. It has been impossible for us to investigate all the paths of research which have been opened up, and prospective workers may be assured, from our own personal experience, that research with stained living cells will amply repay the time and patience expended on it.

For the examination of the *arrangements* of the cells in living tissues we have not, so far, been able to make this *in-vitro* method so useful as is the older method of examining sections of dead tissues, but we think that improvements may be possible. For blood-



examination, on the other hand, it takes one into a different realm compared with the older methods. Examined by the older methods, a cell appeared usually as a flattened, stained diagram; by the new one it appears as a sphere. The difference is comparable to that which exists between an old Japanese print in which there is no perspective and a perfect photograph seen through a stereoscope. By the older methods, for instance, the nucleus of a lymphocyte appears as a flattened, homogeneously stained mass, or perhaps the stained chromatin resembles a "spireme" within the nucleus; by the new method it is seen at a glance that the nucleus in the living cell is a round, transparent ball, studded on its outside by minute chromatin granules. There is no doubt that the observation of the living cell is a new study. In almost every slide one sees something of interest which has not been seen before. Living cells seem to have small points of individuality which can only be seen when they are stained alive.

Take for example the phenomena of cell-division. The mitotic divisions, although the same in general principles (unless of course we take steps to induce asymmetrical divisions by an alkaloid) are almost always slightly different, depending to some extent upon the stage of division reached, and upon the attitude in which the cell happens to be presented to the observer.

By this means of cytological study we may frankly say that we cannot tell what revelations may turn up at any time. This book will record a few of them, but

there are doubtless many more to come. The feeling of astonishment may be imagined when one of us for the first time—and the cells have been discovered for more than a century—saw most of the polynuclear leucocytes in the specimen in the act of division. It was expected, it is true; but the way in which these cells divide was by no means expected.

We have carefully searched the literature relating to our subject, without discovering points which have helped us. Most of the literature is devoted to descriptions of morphology which are not of much assistance in this kind of experimental work. There is no literature dealing with the effects of chemical substances on stained, individual, living human cells, and if a point is to be unravelled we have found it better to make experiments for its solution rather than to depend upon any literature dealing with the observation of dead cells.

The new investigator will have to begin at the beginning, which is not far off, and he will have to do so with an open mind.

The foregoing points indicate briefly the scope of this book descriptive of the new methods, and of the paths of research which have been opened by them. But we shall also describe in detail the main path which *we* have followed—namely, the adoption of the methods for the elucidation of the cause of cancer. It must be obvious that since we can now induce proliferation in human cells, and since the proliferation of certain human cells is the fundamental condition which characterises cancer (for that is what it is), we



can, by investigating the chemical cause of proliferation, throw considerable light on the cause of cancer. Cancer is essentially a growth caused by excessive cell-proliferation, and the new methods are the only ones which have given us the power to induce an individual cell to reproduce itself.

As will be seen later, we can say more than this, for we can induce by certain specific chemical agents those remarkable asymmetrical mitotic divisions in human cells which are characteristic of many of the divisions which occur during malignant proliferation. The latter part of this book will therefore relate to Cancer Research.

Before closing this chapter, two other points must be mentioned. The usual cytological phraseology has been found to be difficult to apply to many of the facts seen by the new methods. For instance, the word "nucleus" has a very vague meaning, and yet every one uses it. It arose, we believe, from the examination of cells with the lower powers of the microscope, which are commonly employed in the study of "pathological specimens." The nucleus of a cell, studied from this aspect, is merely a deeply stained body within the cell; but in reality the nucleus is composed of several different parts, each of which has a separate function during cell-division. The body which appears as the nucleus in some cells has a very different function to that which appears as the nucleus in others. For instance, the body which appears as the nucleus of a lymphocyte under low magnification forms the spindle; whereas what are usually described as the nuclei of

leucocytes are their centrosomes. The so-called nuclei of leucocytes ought, we think, in reality, always to be called the centrosomes, and the word "nucleus" deleted from their morphology. We have done our best to retain the usual cytological terms in the senses in which they are usually employed; but we must ask some indulgence when referring to those cells in which divisions have been seen for the first time, and in which these divisions differ very materially from those which occur in other types of cells. Again, we use the definition "amœboid" for the exaggerated movements exhibited by cells under the influence of alkaloids, but it must be understood that these movements differ from the blunt and sedate amœboid movements which are commonly seen—that is to say, they are far more exaggerated and are absolutely characteristic.

We think that, from the persistent examination of dead structures, cytology has been rather led away into a maze from which it will be difficult to extricate it; and it is possible that pathology may have to be modified in some of its points now that we know a great deal more regarding the causes of the proliferation of cells.

The last point to which attention must be directed is, that one ought to be careful how attempts are made to demonstrate new facts observed by this method to other people. If the specimen is actually under the microscope, and other people are present, then, of course, a few persons can see the new fact. But these living cells never last long, and many has the occasion

been that a few persons have seen, say a beautiful mitotic figure, when suddenly a later arrival at the microscope says that he can see nothing, and on examination it has been found that the figure has completely vanished owing to the onset of achromasia. If other people wish to see any experiment, two or three should await beside the microscope; but they may have to wait a long time before a typical specimen is found, for, as has been pointed out, cells rarely present *exactly* the same appearances every time. It is of common occurrence that on one day perfect specimens continually present themselves, but on the next every cell appears to be distorted, or always in the wrong position. For this reason we have found it better to take photomicrographs and convert them into lantern slides rather than attempt demonstrations to many people.

It is right to mention that this method requires the expenditure of patience and time on the part of the investigator. One cannot attain good results in a few minutes, but if some time is devoted to it the value of this *in-vitro* method will be appreciated.

## CHAPTER II

THE GENERAL PRINCIPLES OF THE METHOD—THE APPARATUS REQUIRED—THE SPECIAL PHOTOMICROGRAPHIC APPARATUS—THE REVOLVING APPARATUS.

THIS method by which cells are observed *in vitro* is very simple. They are placed on a film of agar jelly, which holds in solution any material with which we may wish to experiment. To prepare the film, a drop of molten jelly is poured on to a slide, which is then laid on a level surface until the jelly sets firmly. A drop of the citrate solution in which, say, blood-cells are suspended is then placed upon a cover-glass, which is inverted and allowed to fall flat on the film. It might be thought that the weight of the cover-glass would be sufficient to kill the cells; but they sink into the jelly to some extent, and so become protected. Before this happens, however, they spread out centrifugally from the centre to the periphery of the cover-glass, and if a drop of blood be examined in this way on stain-containing jelly they may be seen by the naked eye rushing in every direction towards the edges of the cover-glass. When this movement has ceased, if the slide is held up between the observer and the

window it will be seen that the surface of the jelly over which the cells have passed is studded with corpuscles.

If the jelly has been properly made the slide may be handled freely. It may be tilted to any angle, and even turned upside down without the cover-glass sliding off or the jelly becoming displaced. This is a fortunate fact, because it enables the microscope to be placed at any convenient angle for examination of the slide or for purposes of photography. If the specimen is quickly focused under the microscope while the spreading-out process of the cells is going on, using a  $\frac{1}{6}$ -inch objective and, say, a No. 4 eye-piece, the picture presented is a very remarkable one. The cells will be seen rushing along in a direction from the centre of the cover-glass towards its margin; they tumble over each other, leucocytes and red cells, lymphocytes and blood-platelets, bumping into each other and apparently all striving to reach some imaginary goal. Gradually the flow becomes slower and slower, the cells cease to "barge" into each other so fiercely, they squeeze past one another, and it will be realized what a marvellous power blood-corpuscles have of accommodating their shapes to almost any requirements.

Leucocytes and red cells all behave in the same way. They allow themselves to be squeezed through gaps between other cells, which appear to be so small that if it had not actually been seen one never would believe it. As the flow becomes slower it will be seen that suddenly a passing leucocyte goes "ashore";

its course is arrested because it has adhered to the jelly, or between the jelly and the cover-glass. Sometimes the rest may be only momentary, when the cell may be seen to revolve on its own axis for a few moments, and then pass on again in the slowing stream. Leucocyte after leucocyte afterwards becomes arrested in this way; they apparently stop first because they are larger and more "sticky." Then the red cells gradually stop, until at last the field is dotted with living blood-corpuscles, which may happen to become arranged in groups or rest singly side by side.

The specimen may now be moved about by means of the mechanical stage, when it will be seen that all the cells in the film of blood under the cover-glass have become arranged in a manner very suitable for examination. The frontispiece of this book is a photomicrograph of a typical field presented by this method.

The living cells all come to rest in a short time, and each one has its own share of jelly-surface, from which it has no alternative but to absorb any substances which have been previously dissolved in the jelly. Having focused a field, therefore, which contains an example of the cell with which one wishes to experiment, it is only necessary to wait until that cell has sufficiently absorbed the contents of the jelly for it to respond to the agent which has been dissolved in it.

"Artefacts" do not exist; the surface of the jelly is the same all over. One has no control over the attitude which a cell may adopt, no matter what part of the jelly-surface it may come to rest upon, nor



over the other cells which form its immediate surroundings. The cells are always placed on the jelly in identically the same way as has just been described, and therefore the only way in which one can *intentionally* affect the individual cells is either by deliberately (1) mixing some other substance with the jelly before it is set on the slide, or (2) by keeping the slide at various temperatures. It sometimes happens that *unintentionally* the cells may become distorted by the presence in their neighbourhood of some foreign substance which has been accidentally mixed with them in the citrate solution in which they have been suspended prior to being placed upon the film; but such a foreign body may easily be recognized.

The apparatus required for these researches is not very elaborate. Many of the earlier experiments were made in a cabin in a battleship, where there is not much room for scientific apparatus, but we simply enumerate them here for the benefit of those who may desire to commence the study of *in-vitro* methods for the first time. They consist of:

1. Microscope slides.
2. Cover-glasses. These should be very thin and  $\frac{3}{4}$  of an inch in diameter. A few larger ones, say  $\frac{7}{8}$  of an inch, may occasionally be needed. A silk handkerchief is required to polish the cover-glasses, which should be very clean and kept in alcohol.
3. Capillary glass tubes. These are constantly in use, and it is well to begin with a stock of 100 of them. They should be about 4 inches long, having an internal



diameter of 2 millimetres, and should be kept in water which has been sterilised.

4. A watch-maker's file for removing the sealed ends of the capillary tubes.

5. Hair-lip pins are most convenient for pricking the finger or the ear to obtain the blood.

6. Two or three needles in handles for teasing out tissues, etc.

7. Pipettes; several 1-cc. pipettes, graduated in 10ths and 100ths; a graduated 10-cc. pipette, and one or two ungraduated of 5-cc., 3-cc. and 2-cc.-capacity.

8. Two beakers. These are used for boiling water in. The jellies are melted and made liquid by immersing the test-tubes containing them in water which is boiling in the beakers.

9. Tripod stand and gauze cover.

10. A Bunsen burner or good spirit-lamp.

11. A 100-cc. graduated measure.

12. Two small flasks.

13. Some glass funnels and filter paper.

14. A selection of test-tubes.

15. A centrifuge.

16. An ordinary chemical *Centigrade* thermometer for recording the room temperature.

17. A good incubator, which should maintain a temperature of  $37^{\circ}$  *Centigrade*, i.e. the temperature of the blood. Hearson's is a very good one, but any of the ordinary water-jacketed types will do. An automatic thermostat is a convenience.

18. The microscope is the most important part of the outfit, and it should be a good one.

This work consists largely of cytology, requiring accurate observation as to details, and the highest powers of magnification. Any good microscope stand will do, but we think that the English tripod one is the best, especially if the special photomicrographic apparatus is adopted, in which case it is almost essential. The larger and heavier the stand the better. It must have a mechanical stage, which should be built with the instrument; not an "attachable" one. The lenses must give good definition. Two objectives only are necessary—a sixth-inch, and an immersion twelfth. We use equivalents of these in a Zeiss D, and a Zeiss 2-mm. apochromatic lens, which is compensated for the long-draw-tube of 250 mm., and which has a numerical aperture of 1.30. There is no doubt that an apochromatic objective for this work is vastly superior to an ordinary twelfth-inch lens, especially if photography is to be used.

The eye-pieces we employ are the No. 4 and No. 8 Zeiss compensated ones, and these, or their equivalents, will be found most useful.

The light should always be artificial; daylight is not suitable for this method. We have found that the inverted incandescent gas-burner gives the best light for ordinary work, or if electricity is preferred, the 1-ampere Nernst lamp is most suitable. If neither gas nor electricity are available, the spirit-lamps which give a light by heating an inverted mantle have proved most suitable in our hands. No matter which light is used, it is better always to use the same, in order that contrasts may be detected readily.

It is well to remember that with this method one cannot afford to waste much time in manipulating the adjustments of the microscope. The cells, under some conditions, die quickly, and we therefore have to search the specimen very rapidly before "achromasia" occurs, when all the cells vanish, as will be presently described. It is better, therefore, to have everything ready before the specimen is prepared.

The microscope should be fitted with a nose-piece, so that the objective can be changed quickly. When using the immersion lens, great care must be exercised in placing the drop of cedar oil on to the cover-glass, for the cells and jelly-films are easily destroyed if it is accidentally touched with the solid oiler. There is neither time nor necessity to reverse the mirror from concave to plane when the objective is being changed from a dry to an immersion one. When searching through the specimens of living cells, rapidity of focusing will be found to be of more value than too much attention to accurate microscopy, which is difficult, if not impossible, to adhere to with this method. The focusing of the substage condenser on the specimen cannot be very accurate. Most microscopes are adjusted for slides of a certain thickness, but *we* have to place a comparatively thick film of jelly on top of the slide, and hence the objective is always farther away from the condenser than it ought to be.

The photomicrographic apparatus (figs. 2-5) invented for this method has been designed so that a photograph can be obtained quickly of any field in

a specimen without disturbing either the microscope or the specimen. Having obtained the negative, the camera is removed in a moment and the examination of the particular cell or specimen under observation can be immediately proceeded with in the usual way. The old forms of cameras which necessitated the moving of the microscope or the specimen are not useful for recording specimens of living cells. An instrument is required capable of being immediately connected with the microscope as it stands, so that two or three records of the same cell may be taken before it dies or becomes achromatic and vanishes. It is necessary to use a powerful light, and the light itself will kill the cells if they are exposed to it for very long. For this reason we employ a powerful light for the photography and another for the eye work, but each of them fixed and capable of being used independently of one another. The inverted gas-burner above referred to, being placed at a distance of two feet above the mirror, gives a soft, indirect illuminant for ordinary work, the other being a powerful electric Nernst burner, which is placed behind (that is, underneath) the mirror. When a photograph is to be taken the mirror is swung aside, and the light from the Nernst lamp replaces that from the gas one.

The microscope is fixed on the bench and tilted at an angle of about  $45^{\circ}$  from the vertical. All the microscopes which we use are bolted permanently on to the bench, and they can only be moved with the aid of a screwdriver. The instruments are not placed vertically, but are tilted at



FIG. 2.—The photomicrographic apparatus. The microscope is ready to be used for direct observation. The gas-burner can just be seen at the lower end of the wooden plank. (N. B.—The sheets of white paper have been placed in this position in this and the next photograph in order to “showup” the apparatus.)





FIG. 3.—The apparatus ready for photography. The mirror is swung aside, and the eye-piece attached to the camera is inserted into the microscope.





an angle, because this is most convenient for *comfortable* use. This last point is most important, for one may have to spend hours searching through films with this method, and it is most wearying to have to work in an uncomfortable position.

Behind the mirror, and standing a little way back from it, there is a Nelson's aplanatic condenser (Watson) with iris diaphragm, and immediately behind this again is fixed a rectangular all-glass water-tank. This small tank has an outlet pipe above, and an inlet pipe below, connected by means of rubber tubes with a sink and a cold-water supply respectively. The water is kept circulating through this tank when the apparatus is in use. Lastly, behind the tank is the burner of a 1-ampere Nernst lamp.

Above the microscope and set at an angle corresponding to its tilt a rigid wooden board is arranged, being fixed to the ceiling above and, by means of a pair of legs on either side of the microscope to the bench below. The board, which is about ten inches in width, by seven-eighths of an inch thick, has a slot cut into it in which a box camera can easily slide up and down and be capable of being fixed at any point by means of a screw clamp. The camera is fitted with a shutter (instantaneous and time exposures) the aperture of which is connected with a "high-power projection eyepiece" (Watson) by means of a flexible velvet collar.

The Nernst burner, the cooling tank, the two condensers, and lastly the camera must all be very carefully centred to the microscope, and immovably fixed so that the whole apparatus may always be ready

for use, the Nernst lamp being kept lighted as well as the gas one during any experimentation in order that a photograph can be taken at a moment's notice. Of course, so long as the mirror is in its usual position no light reaches the specimen from the Nernst lamp; swing the mirror out of its position, and the light is instantly changed from that of the gas-burner to the powerful one from the Nernst burner. The distances between the Nernst lamp, aplanatic condenser, and the substage condenser, are of great importance. It must be determined at the outset by trials which distances give the best results. The presence of the water-tank renders it difficult to make a rule.

When a cell or other object comes under observation which it is desirable to photograph, the working eye-piece is removed from the microscope draw-tube; the camera is allowed to slide down the beam until its shutter is about an inch from the mouth of the draw-tube, when it is clamped to fix its position. The projection eye-piece, which is already attached to the camera-shutter by means of the flexible velvet collar, is inserted into the microscope draw-tube. The mirror is now swung on its gimbals out of the focal axis, thus allowing the light from the 300-candle-power Nernst burner to replace that of the gas-burner; and the former, after being cooled by transmission through the intervening water-trough, is projected directly through the two condensers. The image of the field of the specimen will then be seen on the ground-glass screen at the back of the camera, where it can be rapidly focused.



FIG. 4.—The photomicrographic apparatus. Showing positions of water-cooling tank and Nernst burner. The microscope mirror is in position for direct observation.





FIG. 5.—The photomicrographic apparatus. The microscope mirror is swung aside for photography.





If preferred, focusing may be done with a lens; but in the case of a specimen of blood, the edges of the red cells afford a good indication of its accuracy, for they seem just to disappear when the accurate focus is obtained. When they are out of focus the edges of the cells stand out in high relief. Having obtained the focus—and stress must be laid on this point—the cell or other object is deliberately thrown out of focus to the extent of about  $\frac{1}{600}$ th of a millimetre<sup>1</sup> by screwing down the fine adjustment so as to bring the objective nearer the object. The reason for this is that the cells are resting on a jelly under a cover-glass which is all the time slowly sinking into the jelly, and, of course, carrying the cells with it. The latter, therefore, are sinking out of focus all the time. By deliberately “over-focusing,” when the exposure is actually made the focus will become accurate, and the sinking of the cover-glass compensated for.

The length of the exposure varies with the objective used and the candle-power of the light, which in its turn varies with the voltage. It is best to find the length of the exposure by experiment, but we give about twenty seconds with the apochromatic objective, using “backed” Imperial plates. The water cooling tank cuts out light, but it is very necessary to use it in order to delay death of the cells and the onset of achromasia, both of which are accelerated by heat rays. The tank cuts off some of the heat rays, but allows the passage of the actinic ones. Many specimens

<sup>1</sup> The fine adjustments of most microscopes are graduated to allow of this measurement.

were lost owing to achromasia before the cooling tank was employed.

The photograph having thus been quickly taken, the mirror may again be swung into position, the camera pushed out of the way, and, having inserted the working eye-piece, the examination of the specimen may be proceeded with, or other fields explored. We have taken a negative in fifty seconds with this apparatus, and as many as five negatives have been taken from different fields in a single specimen; but such speed is not often necessary.

All the photographs which illustrate this book have been taken with the apparatus just described. It never gives trouble, and has proved most useful in supplying a means of recording the "specimens." It used to be most annoying to see unique mitotic figures or other interesting specimens slowly vanish before one's eyes without being able to record them satisfactorily. In fact, the best mitotic figure I have ever seen in a lymphocyte was induced before we possessed a camera; and although thousands of figures have been seen since then, I have never seen a picture comparable to it. It was seen by Professor Harvey Gibson as well as by myself.

There is one other useful piece of apparatus which requires mentioning, viz. the "revolving apparatus." This is a simple clock-work contrivance which keeps a long test-tube revolving on its long axis. The test-tube is placed horizontally. The object of the appliance is to keep the blood-cells constantly moving in the "citrate solution," or other medium in which

they may be suspended, while samples are under examination. If the capillary tubes containing the specimen are laid for some time on the table, the corpuscles will sink to the most dependent part of the citrate solution, and will ultimately adhere to the glass. By placing the tubes in the "revolving apparatus" this is effectively prevented. It is a good thing to have in the laboratory, for it delays loss of vitality in the cells; but it is not essential.

## CHAPTER III

### THE PREPARATION OF THE JELLY FILM

AGAR, the substance used for making the film on which the cells are examined, is obtained from seaweed. It is very cheap, and may be bought in strips or as a powder. We have used Merck's powdered agar, which is quite neutral and pure. It is insoluble in cold water, but immediately soluble in boiling water. This solution, therefore, on cooling, sets as a jelly. It is necessary to have a stock of jelly constantly in hand, and a 2-per-cent preparation is used throughout. This will melt when its boiling-point is approached, but will not set again until the temperature has fallen almost to 40° C. Unlike gelatine, this jelly may be boiled over and over again, and it will always set at its usual temperature.

The jelly is made in 2-per-cent strength for the reason that it will stand diluting with its own volume of water or other solution, and will still set as a jelly—that is, a 1-per-cent solution of agar jelly will set on a slide in the form of a film as it cools. By using the 2-per-cent preparation we are enabled to add an equal volume of any solution we please,

so that the result is that the 1-per-cent jelly may contain quite a variety of substances, and if some human cells are placed on its surface we may try the effect on those cells of any of those materials which have been added in solution to the 2-per-cent agar. We are thus able to investigate, by a method which is simplicity itself, the effects of drugs or chemical substances upon the individual human cell.

Before we begin to discuss this subject, however, we must be certain that the cells are alive when they are being subjected to the drug. It is, of course, well known that when, say, a drop of blood is removed from the finger the leucocytes are alive; but it is necessary to be certain that they are not killed immediately they are placed on the jelly-film. As will be discussed at greater length later on, we can always ascertain whether white blood-cells are alive or not by mixing a certain quantity of an alkaloid with the jelly; for alkaloids excite amœboid movements, and it is obvious that these movements cannot occur in a dead cell. Since alkaloids have supplied the means of determining this point, we have also been able to ascertain how to make the jelly so that it will keep the cells alive as long as possible; for it is clear that a jelly which will allow cells to remain excited for the longest period with a given quantity of alkaloid must be the best jelly for keeping the cells alive when made without the alkaloid. The presence of a combination of certain salts is essential.

Suppose a drop of blood is placed on to a film of jelly which contains only agar and water and no



salts. The red cells will hæmolyse immediately. The white cells are worth watching. As soon as they come to rest, or even before that, the polynuclear leucocytes seem to swell up, the granules exhibit "furious" Brownian movements, and in a few moments the cell totters and then bursts. Water kills blood-cells instantly if there are no salts present. Let the experiment be repeated, but, instead of using merely agar and water, now make the jelly with sodium chloride in the strength of "normal saline solution." It can be made thus: Melt a few cubic centimetres of 2-per-cent agar jelly and place 1 cc. in a test-tube. Prepare a solution of 1.8-per-cent sodium chloride in water. To the 1 cc. of molten 2-per-cent agar jelly add 1 cc. of the sodium-chloride solution. The test-tube will now hold 2 cc. of a 1-per-cent agar jelly containing 0.9-per-cent sodium chloride, *i.e.* "normal saline solution." The whole is melted again, and a drop poured on a slide. If some blood is now examined on this jelly, it will be seen that the red cells do not "lake" immediately. The leucocytes, however, again die very quickly, as is seen by their swelling up, the onset of "dancing" movements of the granules, and by rapid bursting, although the rupture will not be quite so rapid as when only water was present.

Now let the experiment be repeated a third time, but instead of adding a solution which contains only sodium chloride, let it contain in addition some sodium citrate, thus: To 1 cc. of 2-per-cent agar jelly add 1 cc. of a solution containing 1.8-per-cent sodium

chloride, and 2-per-cent sodium citrate.<sup>1</sup> (When this jelly is spread on the slide it will contain 1-per-cent agar, 0.9-per-cent sodium chloride, and 1-per-cent sodium citrate.) The picture presented by blood spread on such a film is very different from those in the last two experiments. The red cells are not crenated, but are beautifully spread out. The leucocytes are not dead, but alive and amœboid; no Brownian movements of the granules can be seen, and the cells do not burst; on the contrary, they will live now for an hour or more. It may therefore be said that for the examination of living blood-cells (and it has been found that it is also the case for all cells yet tried) the jelly must always contain a certain amount of the salts sodium citrate and sodium chloride. "Normal saline" is not enough by itself. Cells die immediately when they are resting on a surface which contains only sodium chloride.

These three experiments will prove instructive for the beginner with this jelly method, for they demonstrate how the jelly is prepared. It must be observed that for the purposes of these researches the supply of jelly is always kept as a 2-per-cent solution of agar. When, however, it is placed as a film on the slide, it is always diluted with an equal volume of some other solution, so that the film invariably contains 1 per cent only of agar. It is in the diluting solution (always added in an equal volume) that the salts, and any other substances to be experimented with, are contained, and, obviously, before being added to the agar they must be of twice the required strength so as to be reduced

<sup>1</sup> Potassium oxalate may be substituted for sodium citrate.

to the proper one in the resultant jelly with which the film is made. It is imperative to explain the way in which the jelly is made even at the risk of being verbose. Bear in mind, therefore, that two solutions are required—namely, No. 1, a stock 2-per-cent solution of agar, and No. 2, a solution which contains the other substances the effects of which are to be tried on the cells. Solutions Nos. 1 and 2 are always mixed together in equal parts and then boiled up to form No. 3, from which the jelly-film on the slide is prepared. No. 1 is always the same. No. 2 may contain a variety of substances, but no matter how much of any substance No. 2 may contain, No. 3 will always have half that amount. For example, if one wishes a cell to rest on a jelly containing 1 per cent of morphine one must have 2 per cent of morphine in No. 2, so that when the two solutions are mixed in equal parts the combination, that is No. 3, will contain 1 per cent of morphine.

A word is necessary as to the effects on cells of the agar itself. It appears to be innocuous. We have tried it in strengths double and even four times as great as that contained in the stock solution, without apparently producing any deleterious effect upon the vitality of the cells experimented with.

## CHAPTER IV

### CELLULAR STAINING, DEATH, AND ACHROMASIA

By far the larger number of cells examined in these researches have been blood-cells taken from the finger. The white blood-corpuscles have offered a very interesting study, and since they respond to chemical agents in a way very similar to those observed in several other varieties of cells, and, since they are very easily obtained and can be very carefully watched, it is convenient to describe what we have seen with them. These cells play an important rôle in the phenomenon of healing, and ultimately go to form some of the fixed tissue-cells, especially after an injury has been sustained.

For the examination of blood-cells in *in vitro* it is best first to mix the sample of blood (which should be drawn freshly from the finger) with an equal volume of "citrate solution."<sup>1</sup> The citration of the blood not only prevents it coagulating, but it also keeps the cells alive sometimes for as long as seven days.

The way in which we citrate the blood is as follows: One end of a capillary tube, such as has been described (Chapter II.), is dipped into the citrate solution, some of

<sup>1</sup> Three-per-cent sodium citrate, and 1-per-cent sodium chloride.

which runs up into it. The amount drawn into the tube can, if necessary, be controlled by keeping the finger on the other end. It has been found most serviceable to allow the solution to fill the tube to the extent of about half an inch, and any excess can always be removed by tapping the lower end of the tube upon the table, which causes some of it to run out. Having got a sufficient quantity of citrated solution into the tube, it is run down to one end of it, and a mark is made at the upper limit (or meniscus) with a grease pencil. The fluid is now run along the tube by depressing its other end until its lower meniscus stands at a level of the mark, and a second mark is then made at the upper meniscus, after which the tube is again placed vertically so that its contents runs down to its original position. The finger having been pricked, a drop of blood is squeezed out and at once allowed to run into and mix with the citrated solution in the tube, the greatest care being taken that no air-bubble intervenes between the fluids. The blood should be allowed to run in until the upper meniscus of the mixed fluids reaches the upper mark. Thorough mixing of the blood with the citrated solution is ensured by rocking the tube in such a way that its contents runs from end to end. The mixture in the capillary tube will now consist of equal proportions of blood and citrate solution, and of this a drop is tapped out on to a cover-glass, which is then inverted and allowed to fall on the agar film in the usual way. When tissues are to be examined, a small portion of the growth or normal structure is either teased out or scraped into a little of the citrate solution in

a watch-glass. A drop of the cell-containing mixture is then placed on to the cover-glass and similarly placed upon the jelly.

The citrate solution simply acts as the vehicle in which the cells are kept in a living condition before being placed upon the jelly, and furthermore, by diluting the blood, it reduces the actual number of cells which come to rest in any field of the film. If no citrate solution is used they are apt to become huddled or crowded together owing to their great numbers, and the leucocytes may become completely hemmed in by erythrocytes so that a clear observation of the whole cell cannot be obtained.

We must now pass on to the study of some of the phenomena connected with the staining of the cells, which have been the means of elucidating many cytological details which have led to the correct appreciation of the effects of chemical substances on cells. In this chapter, however, I do not propose to discuss very deeply the actual laws by which the staining of the cells is controlled; that will be reserved for discussion when I come to speak of the diffusion of the substances, including stain, into the cells. In the meantime I shall simply describe what happens to the cell as it absorbs the stain (say Unna's polychrome methylene blue, Grubler); how the stain causes the gradual death of the cell (the staining of the nucleus invariably kills it) and how death is followed by achromasia. The amount of stain which is put into any given jelly is not added in a haphazard way, the actual amount necessary to cause



leucocytes to stain deeply in a given time being a very definite one, as will be described in the next chapter; but in the meantime we must assume that a jelly has been correctly prepared containing, besides the proper proportions of sodium citrate and sodium chloride to keep the cells alive, the proportion of stain requisite to enable us to observe its gradual passage into the leucocytes as they absorb it.

The agar jelly, of course, will be coloured purple owing to the stain it holds in solution, but it will be quite transparent and will allow sufficient light to penetrate it so that the cells may be clearly observed.

Having without delay placed the film, with the blood-cells upon it, under the microscope, at first the cells will be quite unstained, but the white corpuscles may easily be recognized owing to their granulation and size. Let a polymorphonuclear leucocyte be watched. Gradually its granules become tinted a faint red colour (fig. 6) and about the same time amœboid movements may begin. If certain proportions of alkaloid have been added to the jelly, these amœboid movements will be very marked (fig. 7). The staining of the granules becomes deeper and deeper, always maintaining the same bright scarlet colour. In spite of the deepening coloration of the granules, amœboid movements will continue, showing that the cells are alive and that their vitality is apparently unaffected by the staining of their granules. It is not only the polynuclear leucocytes that behave in this way, but the mononuclear, or lymphocyte, cells as well.



FIG. 6.—The granules of the leucocyte are gradually becoming stained. The red cells are unstained. Low power.

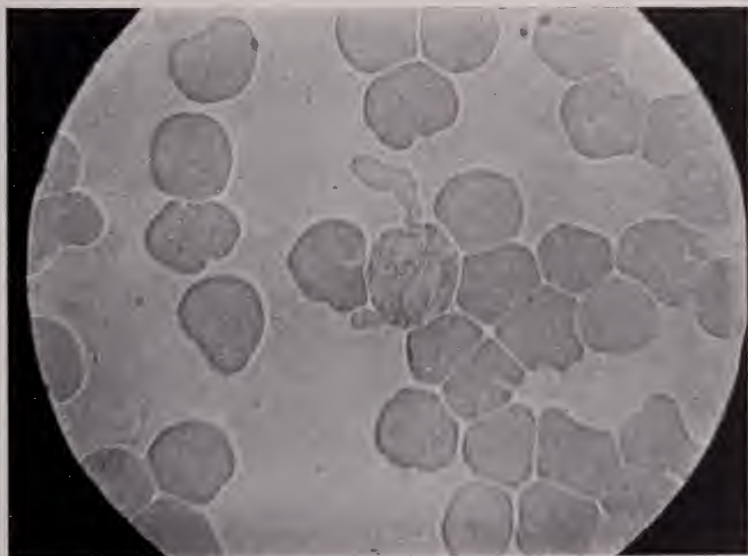


FIG. 7.—The leucocyte's granules are stained. Its nucleus is unstained. The pseudopodia are extruded in response to atropine, which is diffusing into the cell as well as the stain.



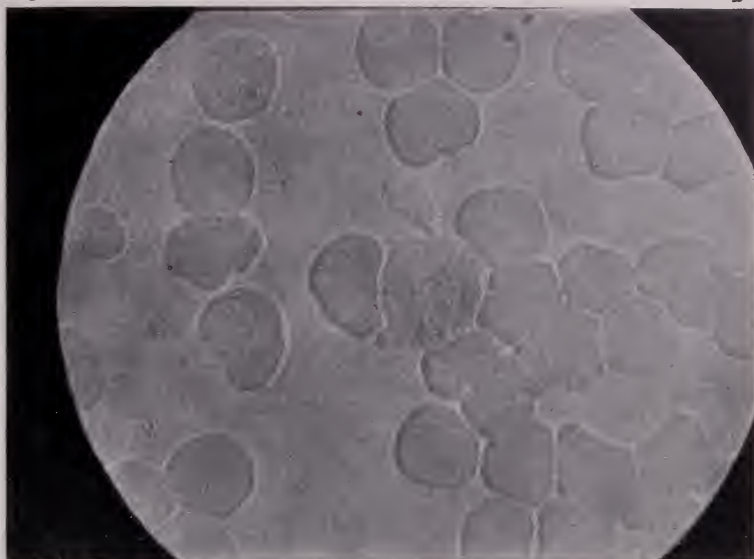


FIG. 8.—The same field as 7. The leucocyte is retracting its pseudopodia.

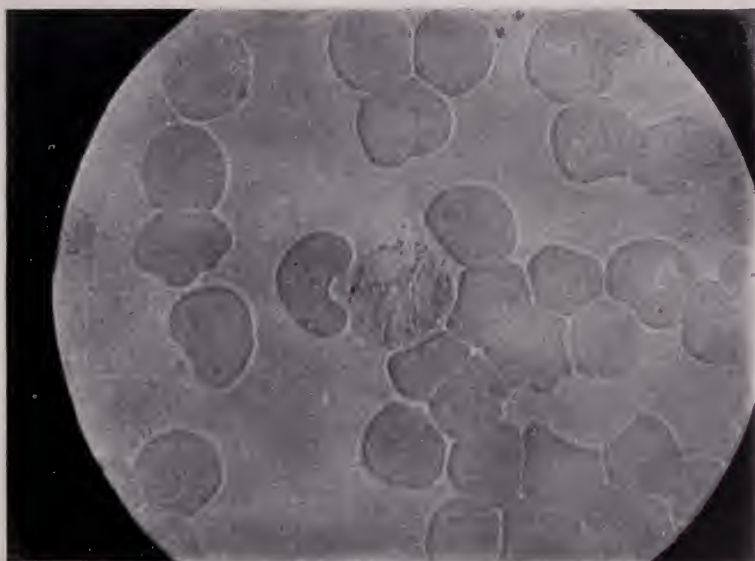


FIG. 9.—The same field as 7 and 8. The retraction of pseudopodia is nearly complete. The lobes of the nucleus of the leucocyte are turning a faint blue colour.





FIG. 10.—A leucocyte excited by atropine. Its granules are deeply stained, and its nucleus is also beginning to stain a blue colour. Low power.

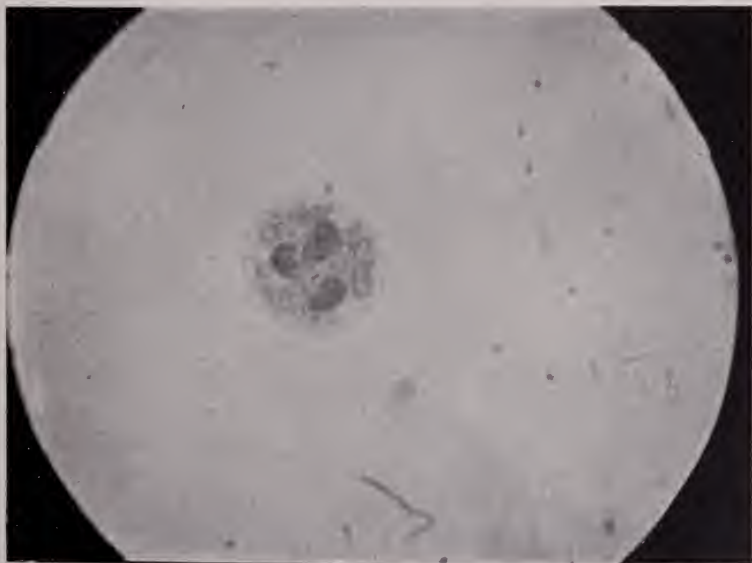


FIG. 11.—A leucocyte which has just been killed by the staining of its nucleus. Its granules are also deeply stained.





After a short time the *extrusion* of pseudopodia ceases, and it will then be noted that general retraction (figs. 8, 9) of pseudopodia begins to occur. In the meantime the lobes of the nuclei of the polynuclear cells begin to turn a faint blue colour (fig. 10). If two or more leucocytes happen to be in the same field, it will be seen that they all behave in a like manner, for the stain affects them all equally. In a few moments all the amœboid movements cease, for death is about to occur, and then, sometimes quite suddenly, the nuclei turn bright scarlet (fig. 11) and the death of the cell takes place.

We have never yet seen a cell show any amœboid movements when its nucleus has stained scarlet. By mixing some blood with a citrated solution of stain one can cause first the granules and then the nuclei of the leucocytes to stain, the difference depending on the length of time the mixture has been made. If now cells with only their granules stained are placed on to a jelly-film which contains an alkaloid, we can excite these cells, showing that they are alive. But if their nuclei are stained scarlet, no excitation or movements of any sort can be produced, and there can be little doubt, therefore, that the staining of the nucleus kills the cells. White blood-corpuscles do not seem to mind the staining of their granules; but the staining of their nuclei invariably causes their death. This is a rule to which we have never yet seen an exception in any cell which we have examined. A stained nucleus is incompatible with life.<sup>1</sup>

<sup>1</sup> We have tried several stains, but this rule holds good with them all.

When the lobes of the nuclei stain scarlet, the chromatin network within them shows up well. The blue coloration which precedes the scarlet one is due, I think, to the staining of the nuclear wall. The polychrome dye contains two stains, a red and a blue one, and the nuclear wall seems to have an affinity for the blue one, while the chromatin combines with the red. The staining of the nucleus, therefore, is a sign that the cell has died, and one now sees a circular dead cell (in reality it is a spherical cell which has become flattened out) with its granules stained scarlet, and in their midst there is the polylobed nucleus, also stained scarlet. Let the specimen be watched still further. Gradually the cell-wall is seen to bulge out in places (fig. 12), apparently away from the granules. After a few moments this bulging becomes general (fig. 13), and the cell presents a clear halo of cell-wall and cytoplasm outside the limit of the mass of granules in its centre. This is due to the gradual liquefaction of the cytoplasm which occurs at death, beginning at the periphery and progressing slowly towards the nucleus. Sometimes a few stained granules appear to migrate by the "dancing" Brownian movement into the liquid cytoplasm which has bulged out the cell-wall. Under suitable conditions the Brownian movement becomes general, showing that all the cytoplasm has liquefied—a certain sign of death.

No matter whether the cytoplasm has completely liquefied or not, however, one of two things is bound to happen after a short time. The granules and nucleus may remain stained for half an hour or so, especially



FIG. 12.—The leucocyte has just died owing to the staining of its nucleus. The cell-wall is beginning to bulge because the cytoplasm is liquefying.

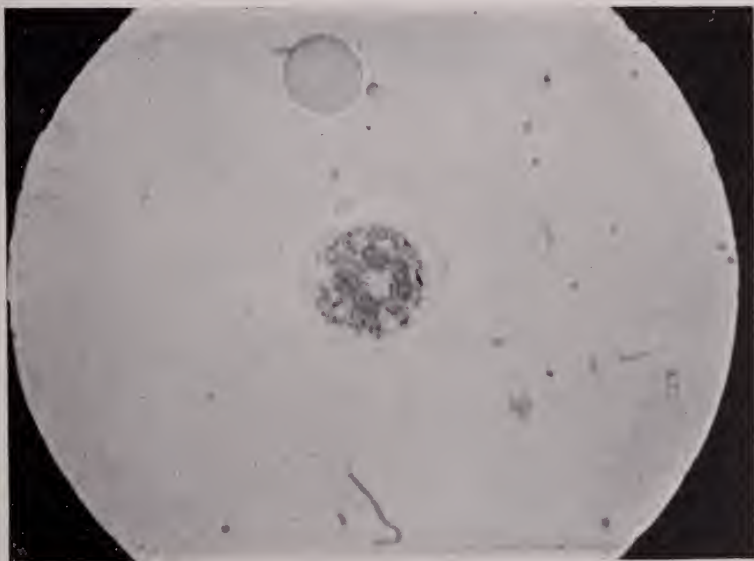


FIG. 13.—The onset of achromasia. The same field as 12. The stain is beginning to fade from the nucleus. The bulging of the cell-wall has become general.



if a temperature of about 30° C. is maintained, which may prevent the bulging of the cell-wall; but after that time the cell will either burst and become achromatic, or become achromatic without bursting (fig. 14). In either case achromasia, or loss of stain from the cell, invariably occurs. If the temperature is low (say that of the room) the cell will probably burst and its granules will be scattered about on the surface of the jelly. Now, when a cell bursts on a jelly which contains salts—such as the one with which we are supposed to be experimenting—there is another rule to which there is no exception, namely, that the cell's nucleus loses its stain *instantly*. In a flash all coloration has gone from it. But the granules may remain stained for half an hour or more; and then they also gradually lose their stain (fig. 15), and appear slowly to vanish from the scene. The phenomenon of achromasia always overtakes the cells sooner or later.

If the cell does not burst, the stain disappears, but its disappearance is much slower. This is a pretty phenomenon to watch; but it requires a warm room or warm stage. Suppose we are watching a cell which is dead, having its nucleus and granules stained bright scarlet. The stain gets a deeper colour, and one wonders how deep a shade it will attain to. Suddenly the staining seems to stop, and the depth of colour may remain the same for a quarter of an hour or so. Then, almost imperceptibly at first, the colour becomes paler, and with an accelerating speed the colour fades away from the lobes of the nucleus, until that structure



remains as unstained as it was when the cell first came to rest on the jelly-film. After a few minutes the granules slowly lose their stain also, until nothing seems to remain. Ultimately the red cells disappear too (figs. 14, 15), and the field, which a short time previously was dotted with red cells and stained leucocytes, now becomes a blank, and a new-comer looking at the specimen would hardly believe that there had ever been any cells under view. The picture afforded by the successive occurrence of the staining, death, and onset of achromasia in the cells is well worth seeing. First the slow diffusion of the stain into the cells, staining first their granules and then their nuclei; the gradual retraction of pseudopodia as the nuclei stain, and then the bright scarlet coloration of the nucleus itself as death occurs. After a pause the gradual fading of the stain, first from the nucleus and then from the granules, until at last nothing remains visible of the leucocyte in the place it filled among the neighbouring red cells. The whole phenomenon reminds one of a lantern dissolving view—the onset of staining, its climax, and then its disappearance. Achromasia invariably occurs after a time—nothing which we know of will prevent it; but heat greatly accelerates its onset, and a ruptured cell always becomes achromatic before a whole one.

I do not propose here to give the details of experiments which I made some years ago, to try to investigate the nature of this phenomenon of achromasia; they will be found in a paper, “On the Cause of Achromasia,” in *The Lancet* of January 23, 1909.



FIG. 14.—Achromasia. The same field as 13. The stain has gone from the nucleus, although the granules are still stained. Note that the red cell is disappearing.

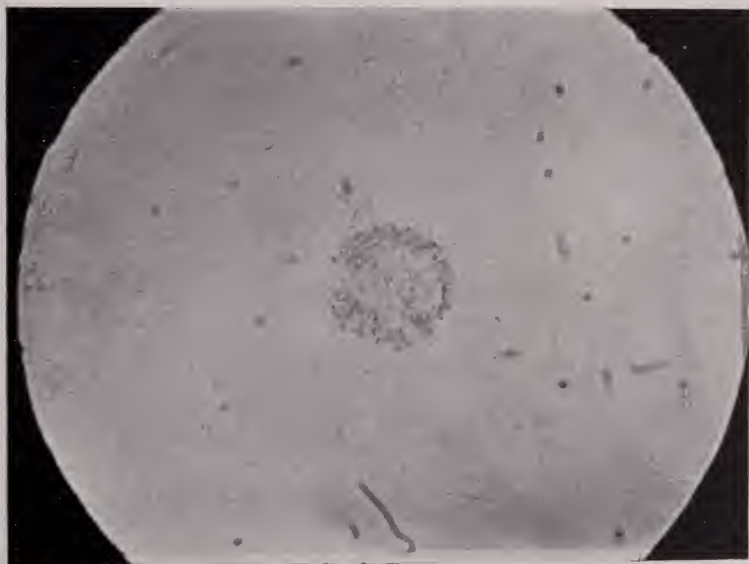


FIG. 15.—Achromasia. The same field as 14. Many of the cell-granules have lost their stain. The cell-wall is nearly invisible. The red cell has disappeared.



I shall now simply state the conclusions which were arrived at then, subsequent experimentation not having altered my opinion in any way.

Achromasia seems to be part of the general disorganization which occurs in a cell after death. I have never seen the phenomenon in a living cell, and one cannot excite an achromatic leucocyte or lymphocyte. It is by no means necessary for a cell to be stained before it can become achromatic; on the contrary, one frequently sees dead cells which refuse to stain, although their living neighbours will stain well under suitable conditions. The rapidity of onset of achromasia depends upon the temperature and the presence and amount of salts. It also appears to depend to some extent on the completion of the liquefaction of the cytoplasm. The more advanced the liquefaction, which, of course, only occurs after death,<sup>1</sup> the more readily does achromasia take place. Heat and salts accelerate it greatly. If there are no salts present, even the nuclei of ruptured cells do not become achromatic for a long time. These stained nuclei may sometimes be seen floating about free from cytoplasm, granules, or cell-wall. I believe that achromasia is due to the chromatin passing out of the dead and liquefied cell by osmosis. If the chromatin is stained, the stain will disappear with the chromatin; if the cell is unstained, it is, of course, impossible to stain its chromatin if the latter has already passed out by osmosis. I believe that this is what has happened in the cell which is commonly

<sup>1</sup> See paper in *Journal of Physiology*, "On the Death of Leucocytes," vol. 37, No. 4, 1908.

known as the hyaline cell. Achromatic lymphocytes resemble them strongly. A dead lymphocyte, from which the granules have disappeared, will not stain, and the cell resting on the jelly looks like a phantom. We have never been able to excite such a cell. If a specimen of fresh blood is placed carefully on a jelly-film, one does not usually see any such cells, for all the cells will stain; but after a while the film may contain many examples of achromatic cells which appear to be exactly like what are known as hyaline leucocytes by the older methods. Achromasia is a certain sign of death, and the recognition of its very characteristic appearances is of the utmost importance in this form of research. It should be borne in mind that a cell with a stained nucleus is dead, and so is a cell which is achromatic.

## CHAPTER V

### THE DIFFUSION OF SUBSTANCES INTO LIVING CELLS— THE “COEFFICIENT OF DIFFUSION”

THE following points regarding the diffusion of substances into cells have been determined by experimentation with this method. A cell does not respond to the action of a chemical substance unless the substance has diffused into it. It is of the utmost importance, therefore, that the laws which are concerned in this diffusion should be understood, both for the practical application of this *in-vitro* method in the study of the actions of substances on individual cells and also, I think, because it throws light upon the way in which drugs produce their effects upon the various systems and organs of the animal body. Very little has hitherto been known concerning the diffusion of substances into individual living cells; and although we do not claim to have advanced the knowledge of the subject to a very great extent as far as its scientific basis is concerned, we can safely say that we are now in a position to cause substances to diffuse into individual cells according to our will. We do not know all the scientific facts, it is true, for more work will be necessary before these can be



determined, but we do know the more important laws which are sufficient for practical purposes.

The diffusion of substances into living cells is purely a physical process. A cell does not seem able to exert any vital control or power of selection whatever over the diffusion of substances into its cytoplasm. In every cell with which we have experimented this has been the case, and if proof is needed it is afforded by the fact that we can at will cause cells to be excited, to reproduce themselves, or to die, by employing the knowledge of the laws, which shall presently be described, enabling us to make substances diffuse into cells at any speed we please.

Owing to a variety of means at our disposal certain cells can be made to die in two minutes or in two hours, whichever one likes, merely by accelerating or delaying the diffusion of the agents into them; and it is clear that if a cell could control this diffusion it would at least make some effort to do so in order to save its own life. They cannot do so, however, and always die with clockwork-like regularity at the end of various given periods of time, which are determined by controlling the diffusion of certain chemical substances into the cell's cytoplasm. As will be shown later, the rate of diffusion of substances into living cells can be calculated by means of a simple equation; and since excitation, reproduction, and death can each in succession be induced *in vitro* by causing the diffusion of substances into cells, it follows that excitation, reproduction, and death may also be induced according to the rules which can be plotted as a simple equation.

Cells, as living entities, cannot refuse to absorb substances, and it is also a rule that they cannot "pick and choose" what they absorb. For instance, a cell cannot take from a solution which surrounds it a protein and refuse an alkaloid. If it is surrounded by both these substances it has to take both. On theoretical grounds, I believe that a solution could be prepared (although we have not yet been able to assure ourselves that such is the case) from which a given cell would be able to absorb *nothing*; but such a contingency, as far as can be seen, would be impossible in the body. A cell does not appear to "feed" in the ordinary sense of the term—that is to say, it cannot seek after food. It has to take what is there according to certain laws, even if it dies in consequence; but it cannot "help itself" in any sense of the term. The life of a cell depends upon substances in its surroundings, even its reproduction depends upon them, and the association between them and its life depends on the diffusion of these substances into the cell itself, which diffusion is in its turn undoubtedly dependent on physical laws over which the cells themselves can individually exert no control. A cell cannot take a cake and leave a bun, so to speak: it has to take a bit of cake and a bit of bun whether it likes them both or not—a law which has been amply confirmed by work extending over a period of five years.

One is open to criticism in this matter; for the objection may be raised that it is well known that some cells of the body are affected by some agents or drugs, while others apparently are not. This

seems to be the case, judging from results; but it does not prove that all the other cells of the body have not also absorbed some of the drug in question as well, and that they may have been affected too, but have shown no signs of this effect. Strychnine, for example, stimulates certain cells of the nervous system, as is shown by the twitchings produced by it. Strychnine also causes amœboid movements in leucocytes—a fact which is easily demonstrated microscopically; but this excitation of amœboid movement within the body gives rise to no symptoms, and of course passes unnoticed. It is important to remember, therefore, that because a certain drug affects certain cells and gives rise to symptoms related to these cells, it does not follow that only those cells have absorbed the drug. All have absorbed a share, but not necessarily to the same extent, as will be shown directly.

A cell, therefore, cannot control the diffusion of substances into itself; but after it has actually absorbed them the protoplasm of different classes of cells seems to treat a substance differently, and the cells may, by this peculiarity of their protoplasm, be able to make use of it, or, on the other hand, they may leave it unchanged, or thirdly, they may have to die from its effects. We shall presently describe how cells can be made to absorb aniline dye which contains two substances—one which causes the cell to reproduce itself, the other a poison which kills it. As both substances diffuse into the cell together, and as the cell cannot control this diffusion, it will respond to both. It will reproduce itself by cell-division in

response to one element, and it will die in the act of mitosis from the effects of the poisonous one. This experiment, which will be described at length later, proves these two points, about which I wish to be emphatic; viz. that a cell cannot control the diffusion of substances into itself, nor can it choose from its surroundings any one substance and leave another. Even at the expense of its life, a cell is bound to absorb from its surroundings any substance which may be present; and this absorption depends entirely upon certain chemical and physical factors.

Before proceeding to describe these laws and factors, other points must be mentioned. We are dealing with *in-vitro* experimentation; and we have no proof that the diffusion of substances into cells *in vitro* is identical with this diffusion into cells *in vivo*. There is, however, strong presumptive evidence that similar conditions prevail. As a matter of fact, apart from the mere phenomenon of diffusion, this possible distinction between the facts learnt from *in-vitro* experimentation and what actually occurs *in vivo* must always be borne in mind in researches of this nature. The force of this point will become apparent later on when we come to deal with induced cell-division; for although one can induce the diffusion of substances into cells or cell-division at will on a microscope slide, it will be seen that these phenomena in the body occur under very different conditions, which must be taken into consideration in forming deductions from *in-vitro* experiments. In the final chapters, however, it will be shown that the results

obtained by *in-vitro* experimentation have been confirmed in some instances by experimentation in the living body, and hence one may, I think, reasonably infer what goes on *in vivo* from what is observed *in vitro*, and that these experiments into individual cells may be undertaken with confidence.

Before continuing this subject another matter connected with it must also be stated. In the previous chapter it was mentioned that cells observed *in vitro* must be resting in a solution or on a jelly which contains certain salts the presence of which are necessary for keeping them alive. In the body one of these salts, sodium chloride, is actually present; but there is no sodium citrate, a solution of which has proved to be the best one for leucocytes and other cells to live in. Obviously in the body there must be some salt or salts for which the sodium citrate is a substitute or equivalent. One of the rôles played by sodium citrate in *in-vitro* experimentation is its property of preventing coagulation of the blood, which seems to be an important one, for related to this is the curious fact that leucocytes will live longer in citrated plasma than in undiluted serum, a point which will be alluded to in the description of the method of measuring the lives of leucocytes. Sodium citrate, however, is detrimental to leucocytes, and there is no solution known which will keep leucocytes or other human cells alive for more than a few days. If there was we should now be in a position to cultivate families of human blood-cells in test-tubes. At present, by means of sodium citrate, one can only make leucocytes "exist" for some hours



while we experiment with them; and it must be borne in mind that since sodium citrate is detrimental, leucocytes or other cells placed in it gradually lose vitality all the time, and that they are under *experimental conditions*.

The laws of diffusion—or rather what we know of them—are simple in their experimental application; but they are difficult to describe.

There are two methods by which it may be known when substances have diffused into a cell. If the diffusing substance consists of a colouring matter which will combine with or otherwise colour the molecules of protoplasm within the cell, one can see the extent of the diffusion by watching the progress of the coloration. The other method consists in the use of a substance which has a specific action on the cell and causes it to give a definite response which will tell us when the substance has diffused in. Of the two methods, the former is obviously the better, for by seeing the gradual staining of the morphological elements of the cell one can more accurately gauge the extent of the diffusion than one can by measuring roughly the degree of a response such as excitation of amœboid movements or even cell-division. It is, of course, possible to employ a combination of both methods, by which much can be learnt; in fact, in this book I shall describe what has been observed, in the first place, by using colouring substances only, afterwards a combination of stain and other substances, and lastly by experimenting with other substances by themselves.

Suppose the jelly on which a given cell is resting



contains a certain quantity of an aniline dye, such as Unna's polychrome methylene blue. This dye combines with the cell-granules and stains them red, and the rate of the diffusion of the dye can be estimated by observing the depth of coloration of the granules and the time occupied before the nucleus stains. The first granules to stain, of course, are those which are nearest to the jelly, for the cell is pressed against it by the cover-glass. With a given quantity of dye, the depth of coloration and the rapidity of the extent of staining will take a certain length of time. No matter how often this experiment is repeated, provided the arrangement of the jelly is always the same, with the same type of cell, the result is always the same; but if a fresh jelly is prepared, with double the quantity of stain, the depth of coloration will be double, and the same extent of staining will be reached doubly as quickly as with the first jelly. If the concentration of the dye is trebled or quadrupled, etc., the depth of coloration and the rapidity of the given extent of staining are also trebled, quadrupled, etc., as the case may be.

Hence we arrive at the first law, which is, that the diffusion of a substance into a cell varies directly with the *concentration* of the substance in the solution in which the cell is resting. The more concentrated the substance, the more it will diffuse into the cell, apparently in arithmetical proportion. In a given time, *ceteris paribus*, a 2-per-cent solution of a substance will have double the effect on a cell as compared with a 1-per-cent solution.

Briefly, therefore, we may say that the diffusion is proportional to the amount of substance diffusing, or we may plot it thus:

$$\text{diff} = S$$

Obviously, the diffusion of a substance into a cell takes *time*. If there is only sufficient dye to combine with a certain amount of protoplasm, the combination will occur in a certain *time*, and then the diffusion will cease, for all the dye will be used up; but if there is a sufficiency of stain for it to go on diffusing indefinitely into the cell until it kills it by staining the nucleus, then the diffusion will go on for a longer time—in fact, it will go on diffusing minute after minute until death occurs. Hence we may say that the longer the time which we observe the diffusion, the greater will that diffusion be, unless the substance is all used up—a contingency which in reality cannot occur in practical experimentation, but it may occur in the body. It must be remembered that once the experimental jelly-film is made it cannot be altered, whereas in the body there can be no doubt that the solutions are being continually modified during metabolism.

With a *given concentration* of dye or other substances in the jelly, therefore, the greater the *time* during which the cell is resting on the jelly, the more of that substance will diffuse into the cell, also in direct arithmetical proportion. Each minute will see an equal amount of substance diffusing, provided the supply of that substance is constant, and that other conditions remain the same during the time.

Conversely, in a *given time*, the greater the *concentration* of the substance diffusing, the more of that substance will pass into the cell, as was shown in the first law. We have now considered two factors, therefore, viz. that diffusion is equal to the *concentration* of the substance and the *time*, or thus:

$$\text{diff} = S + T$$

The next factor to be considered is *heat*. *In vivo*, of course, variations of temperature are not very great, but with *in-vitro* experimentation the temperature must be carefully considered, for we may keep the slide with its jelly-film with which we are working at a variety of temperatures, ranging from that of the room in which one works to that of the blood. Heat increases the diffusion of substances into cells in a marked degree, and this increase is also in arithmetical proportion. Each degree of temperature means a definite increase in the diffusion, and therefore the diffusion can be regulated to a nicety by keeping the slide on which the cells are resting at a definite temperature. Of course if extremes of heat are used death will occur; but within reasonable limits, which are compatible with life, one can employ heat to great advantage in these experiments. *Heat* therefore must be coupled with *concentration* and *time* as a factor which increases diffusion; and our equation now stands thus:

$$\text{diff} = S + T + H$$

There is one other factor which increases the diffusion of substances into cells more than any of the three other

factors already mentioned. *Alkalies* and *alkaline salts* greatly increase the diffusion of other substances into living cells. By means of a strong alkali one can cause a substance like stain to diffuse into a cell so rapidly as to induce death and staining of the nucleus almost *instantly*. And this marked increase of diffusion caused by alkalies also takes place in an arithmetical progression; that is to say, if the jelly or solution contains 2 per cent of an alkali, another substance present will diffuse into the cell twice as rapidly as it would if the jelly or solution only contained 1 per cent of the same alkali. Our equation must therefore contain a symbol for alkali also:

$$\text{diff} = S + T + H + A$$

All the above four factors—namely, the *concentration* of the substance diffusing, the *time*, the *heat*, and the *alkalies*—increase the diffusion. Neutral salts, however, decrease it. The more of a salt one adds to the jelly, the less of any other substance, *ceteris paribus*, will diffuse into the cell in a given time. And this retarding effect of a neutral salt also varies exactly with the amount of the salt present. We may therefore add salts to our equation with a minus sign before them, thus:

$$\text{diff} = S + T + H + A - \text{salts}$$

Acids, of course, delay the diffusion of other substances, for they neutralise alkalies; and the amount of retarding effect due to an acid is in exact proportion to its neutralising effect on any alkali present. But apart from this neutralising action of acids, they also actually

retard diffusion themselves according to their strength; that is to say, that if a certain amount of diffusion of a substance will occur from a neutral jelly, the addition of an acid will delay that diffusion in direct proportion to the amount of acid present. As a matter of fact, acids play only a very small part in these researches, for it has been our endeavor to copy the conditions found in the body as much as possible, and cells do not normally come into contact with acids to a great extent. For this reason, as will be shown later, we actually take steps to eliminate the consideration of acids from our experiments, in order to simplify matters.

The foregoing, then, are the factors which increase or decrease the diffusion of substances into living cells. We have no right, of course, to assert that *all* alkalies increase and all salts retard the diffusion of substances into cells, for we have not tried them all; but as far as we have experimented they seem to obey a general rule. As has already been stated, one can only touch on the main principles of this subject of the passage of substances into individual cells, about which little was known before this jelly method of *in-vitro* staining was invented.

Up to the present I have used the expression "cell" in its widest sense. Cells exist as individuals, and as individuals in classes. One may say that polynuclear neutrophile leucocytes are a class of cell, and that erythrocytes are another class of cell.

The diffusion of substances into cells is generally the same in individuals of a class, but it presents great differences in the various classes. For instance, if a



jelly is suitably prepared to stain the nuclei of leucocytes in a given time, it will stain the nuclei of *all* the leucocytes in that time, and it will always do so. There will, of course, be a few exceptions among individual cells which have died or which have become achromatic, but generally speaking all the cells obey the rules of their class. In some classes of cells, however, such as those of the epidermis, we have not yet succeeded in causing anything to diffuse into them at all; and in some of the larger cells, such as some epithelial cells, only a few types will absorb substances *in vitro*; yet if some of the cells of a class in a specimen will absorb a substance at a certain rate, the others of the same class, which are not achromatic, will also absorb the substance at the same rate. It must therefore be grasped that the individual cells of a class will absorb substances in the same way as each other, and the diffusion into them will be influenced by the usual factors in the same way in each cell of the class; but substances diffuse into the cells of different classes at different rates.

Now we come to an extremely important factor which has not been mentioned before, and which is the last one to be taken into consideration. It is the "coefficient of diffusion."

We may prepare a jelly containing a certain concentration of stain, alkali, and salts which will allow a certain amount of diffusion of the stain into a certain class of cells at a certain temperature in a certain number of minutes. Another class of cells may then be tried on a film made from the same jelly under



the same conditions, when it may be found that now no staining, or less staining, may take place. If one adds more stain or more alkali, or more heat, or allows more time, this second class of cells may then stain. Hence we may say that the second class of cell has a higher "coefficient of diffusion" than the first, for it requires more of one or more factors which increase diffusion to cause a certain extent of diffusion into it than did the first class of cells. Different classes of cells may therefore each have different coefficients of diffusion, but in spite of this fact the diffusion of substances into all classes of them depends on the factors already expressed by the equation:

$$\text{diff} = S + T + H + A - \text{salts.}$$

That is to say, that the factors given in the equation increase or decrease the diffusion of substances into all cells; but some classes of cells require more or less of them to cause the same amount of diffusion than do others.

It is obvious, therefore, that we must always find the coefficient of diffusion of a class of cells before we can attempt to make substances diffuse into them; and we find the coefficient of diffusion by ascertaining the number of the factors expressed in the equation, and the amount of each of them required to cause a certain extent of staining of the cell. By means of the equation we can set down algebraically the number of factors and the amount of each of them required to produce this certain extent of staining; and then they are

all added up to make a grand total figure—which represents the “coefficient of diffusion,” or, to express it briefly, the “*cf*” of the cell.

The coefficient of diffusion of a cell is determined by adding up the total amounts of the factors required to cause a certain *extent of staining* of the cell. The extent of staining which we always use as a standard is the *staining of the nucleus*. Now, the “moment” of the staining of the nucleus of a cell can be recognized through the microscope, and it has an additional importance, insomuch as it is coincident with and signifies the death of the cell. In reality, therefore, the determination of the coefficient of diffusion of a cell, as well as supplying the rate of diffusion of substances into it, also tells us how much of the stain, together with the other associated factors, are required to make it (the stain) diffuse into the cell so as to cause the cell’s death *in vitro*. In other words, it tells us the amount of a standard dye required to be in the immediate surroundings of a cell, so that it may diffuse into it to such an extent as to cause its death by combining with the chromatin within the nucleus.

In order to determine the coefficient of diffusion of a cell, however, it is necessary to count up, not only the number of the factors required to cause staining of the nucleus, but also the *amount* of each factor required. To do this it is necessary to measure each factor. One could, of course, measure the chemical factors, such as *alkalies*, *salts*, etc., in grammes, the *heat* in degrees of temperature, and the *time* in seconds; but this would necessitate a complicated total figure involving grammes,

degrees, and seconds. It has been found preferable to measure these factors in special units which can, if necessary, be resolved into their proper ones of grammes, degrees, and seconds.

For instance, in order to remember the rate of staining of a class of cells it would be most inconvenient to have to make a note of a statement such as this: To stain the nuclei in twenty minutes, it is necessary to keep the cells at 20° C. on a film made from a jelly containing 0.5 cc. of Unna's stain, 0.16 gramme of sodium chloride, 0.03 gramme of sodium citrate, and 0.3 cc. of a 5-per-cent solution of sodium bicarbonate. It is much simpler to say that the jelly contains so many "units" of stain, salts and alkali, heat and time. One may go farther and express these units as a simple equation, thus:

$$cf = \overset{\text{Stain.}}{(5s} + \overset{\text{Alkali.}}{3a} + \overset{\text{Heat.}}{3h} + \overset{\text{Time.}}{2t}) - \overset{\text{Slats.}}{(c + 2n)}.$$

A letter by itself means one unit of the factor; a number before a letter means that there is that number of units of the factor:  $c$  means a unit of sodium citrate,  $3c$  would mean three units of it, and so on.

It will be grasped that it is better to make "one unit" of any factor a standard quantity, and these quantities have been chosen with a special object. As has been previously explained, the coefficient of diffusion of a cell is the total number of units of the factors required to cause staining of the nucleus. Some of the factors increase the diffusion into the cell, and others decrease it. A unit of a factor which increases diffusion is so chosen that the increase it causes is equal to

that of one unit of any other factor which also increases diffusion. Likewise a unit of any factor which retards diffusion is also equal to a unit of any other factor which does the same thing. But further still, a unit which increases the diffusion of a substance into a cell is so chosen that the increase which it causes can be exactly neutralised by a unit of a factor which retards diffusion. The units are all equal in value, so to speak. Some increase diffusion, and some decrease it. Any number of units of factors which decrease diffusion retard exactly the increase of diffusion due to the same number of units of factors which cause increase of diffusion.

By the first law, if we double the quantity of the dye in the jelly, we double the rapidity of its diffusion into the cells. A convenient quantity was chosen, namely, 0.1 cc., and this contained in 10 cc. of jelly constitutes *one unit* of polychrome dye.<sup>1</sup> Let us suppose that this quantity (one unit) causes staining of the nucleus of a given cell in a certain time. If now another unit is tried, the cell will stain in half the time it did before.

The alkali, sodium bicarbonate, increases the diffusion of other substances into cells, and therefore it greatly increases the rapidity of the staining by polychrome methylene blue. Now, since all units must be equal in value, it was ascertained experimentally that 0.1 cc. of a 5-per-cent solution of sodium bicarbonate exactly doubled the rapidity of diffusion of one unit of

<sup>1</sup> Unna's polychrome methylene blue (Grübler) is only supplied in solution, which is standardised. It cannot be made in a powder.

polychrome dye. Hence the unit of alkali is 0.1 cc. of a 5-per-cent solution of sodium bicarbonate.

Time is a factor. One unit of time is ten minutes; and since time increases diffusion in arithmetical proportion, therefore in twenty minutes (two units) the diffusion of one or more units of the dye or other substance will be doubled.

The unit of heat is  $5^{\circ}$  C.; unity is  $10^{\circ}$  C., because one cannot conveniently work at a temperature below this point;  $20^{\circ}$  C. is three units, etc.

Salts delay diffusion. The two commonly employed are sodium citrate and sodium chloride. Their units respectively are 0.03 gramme and 0.08 gramme. One unit of sodium citrate or sodium chloride will prevent the increase of diffusion due to one unit of alkali, heat, or time; an extra unit of stain will neutralise a unit of salt.

Hence the units of all the factors are so measured experimentally that they are as nearly as possible equal in value. The delay in diffusion caused by a unit of a substance which decreases diffusion is equal to the acceleration occasioned by one which increases diffusion. It will therefore be realised that the units can be substituted for each other. A unit of alkali will double the diffusion of the dye, but so will another unit of the dye itself. Again, the unit of time is ten minutes; since time increases the diffusion by arithmetical progression, another ten minutes of time is equal to a unit of alkali or another unit of dye. Salts delay diffusion; we have found out how much of a salt, such as sodium citrate, is required to decrease this diffusion in equal

proportion to the increase caused by one unit of alkali, dye, or time. All the units are equal to each other as regards the increase or decrease of diffusion, and therefore they are interchangeable. Hence we may simplify our equation by adding together all the units of all the factors and making a grand total of them; thus:

$$cf = (5s + 3a + 3h + 2t) - (c + 2n) = 13 - 3,$$

or, simpler still:

$$cf = 10.$$

This method of determining the coefficient of diffusion is intended principally to assist experimentation with these *in-vitro* technics. The act of its determination gives up the comparative rate of the diffusion of other substances into the cells under observation, and tells us how to prepare jellies for further experimentation with these substances. For practical purposes, the equation and the measurements of the units of the several factors (which are used continually, not only in the initial determination of the coefficient of diffusion, but in all subsequent experimentation) have been devised with a view to the simplification of the practical methods to be described in the next chapter, where full details for the preparation of the jellies, etc., will be stated. These laws of diffusion were ascertained in the first instance by me with the jelly method as described in the paper in the *Journal of Physiology* already referred to, and they soon led to the method of determining the coefficient of diffusion by the same method which was published in a paper in the *Proceedings of the Royal Society*



(B. Vol. 81); and the description of the methods and laws given herein are in reality an elaboration of the original ones given in the papers mentioned. Much work has been done, however, since those papers were written, including induced cell-division by a variety of chemical substances, and all of it has been carried out with those specifications as bases. The point is mentioned in order to show that the method is fairly reliable. New technics of this nature, where one is dealing with a series of factors, all of which are variables, are prone to become involved in their application. The "moment" of the staining of the nucleus cannot be a very accurate one, and the more factors and units one deals with, the more do small errors assert themselves.

It is a simple matter to note the effects on a cell of two or three units of a dye and a unit or two of alkali. But when one deals with complicated equations involving twenty or thirty variable units, each of which modifies the action of its neighbour, it sometimes follows that complicated situations arise. For instance, the units of the two salts are satisfactory when small quantities of them are used; but with larger quantities it will be found that they are not quite so accurate. For practical purposes, however, the units given have been found to be sufficiently useful; but if this method was to be employed to determine the more scientific data of the action of the several physical factors in increasing and decreasing diffusion, I am prepared to admit that some units will require modification.

## CHAPTER VI

### THE PRACTICAL DETERMINATION OF THE "COEFFICIENT OF DIFFUSION OF CELLS," AND ITS APPLICATION TO THIS IN-VITRO METHOD OF RESEARCH

IN the foregoing chapter I endeavoured to give an outline of the principles of diffusion of substance into individual cells, and the method of the determination of the coefficient of diffusion. In the present chapter I shall describe, in detail, how those principles are applied experimentally, and how one can find out the coefficient of diffusion of a given class of cells. The preparation of the jellies from which the films are made constitutes the most important part of the procedure. The chemical substances which are to be made to diffuse into the cells are contained in the jelly together with the other chemical factors, which increase or decrease diffusion. The factor *heat* is measured by keeping the slide on which the jelly-film is set at a certain temperature, and the length of time the slide is kept at this temperature determines the amount of the factor *time*. The coefficient of diffusion of a cell, as already pointed out, is arrived at by

ascertaining experimentally the lowest sum of units of the factors—both chemical in the jelly and physical as applied to the slide—which will just cause the cell's nucleus to stain. In the original paper, already referred to, which specified this method and the coefficients of diffusion, the following definitions were given:

When a film of agar jelly contains stain and other substances, its Index of Diffusion ( $f_x$ ) may be defined as the sum of its constituents, which delay diffusion subtracted from the sum of its constituents which accelerate diffusion added to the quantity of stain contained in the jelly.

The Coefficient of Diffusion ( $cf$ ) of a cell is that Index of Diffusion plus the time and temperature required to cause staining of the nucleus, or staining of the cytoplasm in unnucleated cells (*e.g.* red corpuscles), when the specimen is prepared by a standard method.

It should be noted that the index of diffusion refers to the composition of the jelly, and that the coefficient of diffusion refers to the rate at which the cell absorbs substances from the jelly.

The standard method of placing the cells on the jelly-film and the general principles of preparing the film have already been described. The cells are mixed with a little "citrate solution" (3-per-cent sodium citrate and 1-per-cent sodium chloride), which acts as a vehicle to keep them alive, and in which they are placed on the cover-glass. Since this citrate solution spreads to the periphery of the cover-glass, it does

not materially influence the diffusion of the stain from the jelly into the cells. When experimenting with blood-cells the blood is mixed with an equal volume of the solution. In the case of other cells the mixture is made as may be convenient. In some instances, when the cells are naturally suspended in a fluid—such as pleuritic fluid—it is unnecessary to use any citrate solution at all, and the cells may be placed, suspended in their own fluid, straight on to the cover-glass.

The general principle of preparing the jelly-film, as given in Chapter III., may be recalled. A 2-per-cent solution of agar in water forms a jelly basis for these experiments. This jelly is kept stored in large test-tubes, so that small quantities of it may be used without having to melt it in bulk every time some is wanted; and it should be filtered when it is made in a manner similar to that employed for the preparation of “nutrient agar,” although, of course, it has no “nutrient” ingredients added to it.

As already mentioned, the 2-per-cent solution of agar has such a consistency that it can, when melted, be diluted with an equal volume of a liquid and yet will set as a firm jelly on a slide when it cools.

Experimentation with this method is essentially a process by which one contrasts the effects of one substance on cells compared with those of others; hence it is important that all the conditions must be the same in each experiment, except the actual difference in the amount of the substance which has to pass into the cells. To this end the jelly-basis is always the same in

every way, and each film is always made from a tube containing 10 cc. of this jelly. The substances which are to be tried on the cells are added to the jelly in the form of the Solution 2 (see Chapter III.), which in its turn is added to the Solution 1. The combination (Solution 3) is always in the quantity of 10 cc., and the film is prepared from this.

It has already been shown that the jelly-film must always contain certain quantities of the salts sodium citrate and sodium chloride, or the cells will not live on it. These salts are therefore added to the jelly-basis or Solution 1. They are added to it in bulk, so that any portion of it contains them, and, in consequence, it is in a condition to cause cells to live on it for as long as possible.

The jelly is prepared as follows:

In a beaker of water stand several of the large test-tubes which contain the stock 2-per-cent agar jelly. The amount required will be at least 50 cc. The water in the beaker should be heated until it boils, when the jelly in the test-tube will be melted.

1 gramme of sodium citrate and then 0.8 gramme of sodium chloride should be weighed out accurately. The two salts are then placed in a flask, which should be of such a size that it also can be steeped in the beaker of boiling water; 49 cc. of the molten 2-per-cent agar solution from the test-tube are now measured out and poured into the flask. The salts slowly dissolve in the molten agar, and, while this solution is taking place, the flask should be steeped in the boiling water in order to keep the jelly molten.

It is important that the sodium citrate should be neutral. Sodium citrate is inclined to become alkaline when exposed for long to the air, owing to the deposit of sodium carbonate. The jelly in the flask, therefore, must be tested and neutralised to litmus with citric acid.

Previous to melting the jelly solution, a solution containing 8.3 per cent of citric acid should have been prepared; and now 1 cc. of that solution is added to the 49 cc. of the molten agar solution in the flask. This renders the whole of the jelly acid, the reason for which will be given directly.

A series of ten clean test-tubes should be ready, and with a pipette 5 cc. of the acid jelly with its salts in solution is measured into each test-tube. Each of the ten test-tubes now contains 5 cc. of the jelly: total 50 cc. in all. The 10 test-tubes are placed in a stand until the jelly is set, and a plug of wool is placed in each; otherwise moulds may grow on the jelly, as it contains salts.

Every one of the 10 test-tubes contains 5 cc. of a 2-per-cent agar jelly, which is acid, because it contains in solution 0.0083 gramme of citric acid. It also contains 0.1 gramme of sodium citrate and 0.8 gramme of sodium chloride; and these tubes of jelly are known for convenience as tubes of “coefficient jelly.”

To any one of these tubes we may add 5 more cc. of any solution or solutions; and if the whole is boiled and mixed by shaking, any portion of the 10 cc. of jelly mixture now contained in the test-tube will set on a slide as a firm jelly-film when it cools.



Since it is essential that all jellies must be alike in all respects except in the actual quantities of the chemical substances which are to be tested on the cells, it follows that every jelly-film is *always* made from 10 cc. of jelly. A film is never made direct from a tube of 5 cc. of "coefficient jelly" unless it previously has had added to it an equal quantity (5 cc.) of some solution. If this rule is followed, every jelly-film will be identical in that the strength of the agar will be the same, and the initial strength of the salts and acid will be the same; but since the second 5 cc. may be composed of any solution, each 10 cc. of jelly may also contain a variety of other substances.

It is in the extra 5 cc. of solution or solutions that the chemical substances, with which one wishes to experiment on the cells, and any chemical factors additional to those already contained in the "coefficient jelly" which are required to increase or decrease diffusion, are added to that "coefficient jelly."

The chemical factors, therefore, such as alkalies and salts, which increase or decrease diffusion assist to constitute the second 5 cc. of jelly which is always added to the 5 cc. of "coefficient jelly." Now, one could, of course, weigh out the right number of units of each factor for every experiment, but it is much simpler to add them from standard solutions. These standard solutions should be kept ready to hand in flasks, on the labels of which should appear the exact amount of each which constitutes one unit.

The same may be said of the chemical substances the action of which one wishes to try on the cells.

For instance, in the determination of the coefficient of diffusion, the stain, as well as the alkali, is kept in standard solution, and is added to the 5 cc. of "coefficient jelly"; but it is most important to remember that no matter how many units of each factor or substance may be contained in the 5 cc. of solution added to the 5 cc. of "coefficient jelly," the former solution must never be more nor less than 5 cc. Therefore, every jelly-film on the slide is always made from 10 cc. of jelly, which in its turn is composed of 5 cc. of "coefficient jelly" and 5 cc. of another solution bearing the units of the chemical factors. No matter how many units of no matter how many factors the second 5 cc. of solution contains, it is always added in the quantity of 5 cc.—no more and no less. Hence, the Solution 3, from which the film is prepared, will invariably consist of 10 cc. Solution 2 may contain one unit of one factor, or it may contain any number of units of any of the factors.

If all the units of the contained factors exactly amount to 5 cc., all well and good; but if they do not do so, the balance *must* be made up to 5 cc. with water. By this means there will always be 10 cc. in the tube of jelly used for an experiment, but it may contain a great variety of units of the chemical factors which increase or decrease diffusion.

The standard solutions of the several factors must be prepared with due regard to this rule. They must not be too dilute or their total may exceed 5 cc. The following list (abridged from the original paper on the "Coefficient of Diffusion") gives not only the actual

units of the several factors, but also convenient standard solutions of them. It is useful to keep this list ready to hand in the laboratory.

**Alkali** (sodium bicarbonate) increases diffusion.—Unit 0.005 gramme. Standard solution 5 per cent, unity being 0.1 cc. It is convenient to remember that this solution is neutralised by a 4.175-per-cent solution of citric acid, and that 1 unit of alkali is neutralised by 0.1 cc. of such a solution. Since the agar at the outset is acid to the extent of 0.083 gramme to 50 cc., a tube of 10 cc., made up as described, must contain 0.0083 gramme of acid. This is exactly neutralised by 0.2 cc. of the standard alkali solution; that is, the agar at the outset, before any stain or other factor is added, delays diffusion to the extent of 2 units. Or, the addition of 2 units of sodium bicarbonate will render the agar neutral.

**Sodium Citrate** delays diffusion.—Unit 0.03 gramme. Standard solution 10 per cent, 0.3 cc. being unity. Since 50 cc. of agar contains 1 gramme at the outset, the 10 cc. of jelly may be said to contain about 3 units.

**Sodium Chloride** delays diffusion.—Unit 0.08 gramme. Standard solution 10 per cent, unity being 0.8 cc. The 10 cc. of jelly contains this from the outset.

**Heat** hastens diffusion.—Each unit 5° C.; 10° C. is unity, 15° C. is 2 units, 20° C. 3 units, etc. For practical purposes I call 37° C. 7 units.

**Time** increases diffusion.—Ten minutes is 1 unit, twenty minutes 2 units, and so on.

Stain, Unna's polychrome methylene blue (Grubler), behaves as if it increased diffusion.—Unit 0.1 cc.

The reason why the "coefficient jelly" is made acid at the outset is this. Alkalies increase diffusion; acids delay it. Acids neutralise alkalies, and between the two there is a neutral point. If the jelly is neutral at the outset, we might have to add acid in the case of a cell having a very low coefficient of diffusion. Again, we may frequently have cases of cells which stain on a neutral jelly. Our equation, therefore, would have to include these three factors—alkalies, acids, and a neutral point—which would be very complicated, as the neutral point would introduce zero. The object throughout has been to make the determination of coefficient of diffusion of cells, and the estimation of diffusion of substances into them, as simple as possible in their practical application, and in order to do this the "coefficient jelly" is rendered acid at the outset and one deals only with the one factor—alkali. The original 50 cc. of jelly contains 0.083 gramme of citric acid; therefore each tube of 5 cc. of "coefficient jelly" contains 0.0083 gramme of citric acid; and each tube will ultimately be made up to 10 cc., which, of course, will also contain this amount of citric acid, unless it is neutralised by alkali. This 0.083 gramme of citric acid represents 2 units of acid, and it is neutralised by 2 units of alkali. If we want to try a jelly which is acid to the extent of 2 units, we simply add no alkali. We are not likely to want a jelly which is

more acid than this, for we have never yet seen any cell stain on a jelly which is acid beyond the extent of 1 unit. We must remember all along, however, that the jelly at the outset *is acid* to the extent of 2 units, and then go ahead with alkali. If we add to Solution No. 2 10 units of alkali, we *say* that the jelly contains 10 units; but in reality it is only alkaline to the extent of 8 units, for two of them have been utilised in neutralising the original 2 units of acid. Of course, the neutralisation of the acid increases the content of sodium citrate to a slight extent, but it is so small that it can be neglected. As a matter of fact, by saying that the content of sodium citrate is usually 3 units, which is in excess of the reality, we compensate for the extra salt produced by neutralisation of the acid. The neutral point we ignore. If a jelly contains only 2 units of alkali, it is in reality neutral; but we need not trouble about that. There is no neutral point in the equation, nor is there a symbol for acid; yet the neutral point and acid both exist in the equation, for the symbol “2a” means neutrality; and the symbol “a” means 1 unit of acid, whereas the absence of the symbol “a” means 2 units of acid.

To recapitulate: Acids and the neutral point are omitted from the equation, but the jelly is acid at the outset, and we deal only with alkali. If a jelly contains only 2 units of alkali, that jelly is neutral. If a jelly contains 15 units of alkali, it really is only alkaline to the extent of 13 units. The jelly basis with which we work is known as “coefficient jelly.”

It is kept in quantities of 5 cc. in test-tubes ready to hand. Each "coefficient jelly" contains sufficient salts for cells to live on it; it is acid to the extent of 2 units; and another 5 cc. of some solution must be added to it before it is poured on to a slide to make the "jelly-film." The film is always made from 10 cc. of jelly.

In experimenting with a certain class of cells, one must in the first instance always estimate their coefficient of diffusion. The cells are mixed with "citrate solution" and kept ready at the room temperature, preferably in the revolving apparatus (see Chapter II.).

In order to determine the coefficient of diffusion of these cells a tube of "coefficient jelly" is taken and a few units of stain are added to it, together with 2 or 3 units of alkali solution. The content of the tube is then completed up to 10 cc. with water. The tube is steeped in the beaker of boiling water until the "coefficient jelly" all melts, when the stain and alkali become mixed with it, as will be presently described. A film is prepared from it on a slide, and a drop of the citrate solution, with the cells in suspension, placed on to it under a cover-glass. The specimen is kept at a certain temperature—representing a certain number of units of heat—until a certain number of minutes—representing a certain number of units of time—have elapsed, and then the specimen is examined under the microscope. If the nuclei of the cells are not yet stained, a few more minutes (*e.g.* another unit of time—ten minutes) are allowed. If, then, the nuclei are not stained,



a fresh film is made from the same jelly, and it is kept at a higher temperature—or so many more units of heat—and again examined. If again it is found that the nuclei are not stained at the end of, say, half an hour, a fresh jelly is made, but with more units of alkali or stain, or both, added to a fresh Solution 2, which is added to a fresh tube of “coefficient jelly.” If the nuclei still again remain unstained, one must try more units of time and more units of heat again. Thus we can go on trying fresh jellies, each of which contains more units of alkali, or of stain; and we try each jelly for a few minutes, first at a low temperature and then with a few more units of heat, until at last we find that the nuclei of the cells are just beginning to stain. The number of units of stain, alkali, heat, time, etc., of each film is noted on a piece of paper, and therefore there is no difficulty in knowing exactly how many units the jelly contained which was instrumental in staining the nuclei of the cells. The units of this jelly are then written out in the form of an equation, and those which retard diffusion—*i.e.* the units of the salts—are subtracted from those which increase diffusion, the difference being the number which is the coefficient of diffusion of the class of cells experimented with.

*Examples.*—We wish to find the *c<sub>f</sub>* of the neutrophile polynuclear leucocyte. A small quantity of citrate solution is drawn up into a capillary tube, as already described, and, the finger having been pricked and a small bead of blood squeezed out, an equal volume of blood is added to the citrate solution in

the capillary tube, which is placed in the "revolving apparatus."

Take a test-tube of 5 cc. of "coefficient jelly," which of course, being cold, is set in the bottom of the tube. Add to it 0.4 cc. (4 units) of Unna's stain; 0.2 cc. (2 units) of alkali solution. Then the tube must have added to it 4.4 cc. of water, to make its total contents up to 10 cc. The colorless "coefficient jelly" will be set at the bottom of the tube, and above this will be 5 cc. of the mixture of stain, alkali, and water. The test-tube is then steeped in boiling water, when the jelly melts, and, as it does so, the stain, alkali, and water pervade the whole of its contents of 10 cc. In reality this 10 cc. of molten jelly is neutral, for the 2 units of alkali have just neutralized the original acidity of the "coefficient jelly." When all is melted and mixed, the tube is taken out of the boiling water, and the contents are actually boiled, until they froth up in the tube, by holding the end of the tube in the flame for a few minutes. A drop of the boiling, stained mixture is then run on to the slide. Here it will set firmly in about three minutes, and if it is held up to the light the jelly-film will be found to be quite translucent. A clean cover-glass is prepared, and a drop of the citrated blood is tapped out of the capillary tube on to it. The size of the drop is immaterial. The cover-glass is taken up between the finger and thumb, inverted so that the drop of fluid is undermost, and it is allowed to fall flat on to the agar-film on the slide. The blood spreads over the film under the cover-glass, and the slide is then placed in the 37° C. incubator

(7 units of heat) for 10 minutes (1 unit of time). The index of diffusion of the jelly is this:

$$fx = (4s + 2a) - (3c + n),$$

where  $s$  = unit of stain,  $a$  = unit of alkali,  $c$  = unit of sodium citrate, and  $n$  = unit of sodium chloride.

At the expiry of the ten minutes the specimen is examined, when it will be seen that the lymphocytes are quite unstained; but the *granules* of the polynuclear leucocytes are just beginning to colour red. To find the  $cf$  of these cells, however, it is stipulated that their *nuclei* should just stain. The specimen is therefore replaced into the incubator for a further ten minutes. Now it will be found that the nuclei of the eosinophile leucocytes are just staining. Hence, although this jelly has not yet given us the coefficient of diffusion of the neutrophile leucocyte, it has determined that of the eosinophile cell, which may be set down as follows:

$$\begin{array}{c} \text{Eosinophile leucocytes} \\ cf = (4s + 2a + 7h + 2t) - (3c + n) = 11. \end{array}$$

where  $h$  = unit of heat and  $t$  = unit of time.

In order to stain the nuclei of the neutrophile cells, we could place the same specimen for another ten minutes in the incubator; but it is not a very safe thing to do, for the cells by this time may be dead. It is better to make a fresh film from another jelly which contains more units of a factor which increases diffusion. We may add more stain or more alkali. Let us try another unit of each, thus: To a fresh tube

of 5 cc. of "coefficient jelly" add 5 units (0.5 cc.) of stain, 3 units of alkali (0.3 cc. of a 5-per-cent sodium bicarbonate solution), and 4.2 cc. of water to make it up to the 10 cc. of jelly. The film is prepared as before, and it is incubated at 37° C. for 10 minutes. On examination, it will be seen that the nuclei of the neutrophile cells are just turning scarlet. Hence this jelly at 37° C. in 10 minutes has the right Index of Diffusion for the coefficient of diffusion of neutrophile leucocytes. The equation may be thus set down:

$$\overset{\text{Neutrophile leucocytes}}{cf} = (5s + 3a + 7h + t) - (3c + n) = 12.$$

The lymphocytes have a *cf* of 14 (2 units higher than that of the neutrophile cells). We may cause their nuclei<sup>1</sup> to stain in 10 minutes at 37° C. by using a jelly similar to the last one, but by either increasing the amount of alkali by 2 units, or by increasing the concentration of the stain by 2 units, or by increasing the alkali by 1 unit and the stain by 1 unit; or by increasing the time by 2 units. Let us try a jelly which contains 2 more units of stain, for now the chromatin of the cells will stain deeply and show up well. The jelly is made thus: To a tube of 5 cc. of "coefficient jelly" add 7 units (0.7 cc. of stain), 3 units of alkali, and, since we now have more stain, only 4 cc. of water is needed to make the contents of the tube up to 10 cc. The whole mixture is boiled and a drop of it spread on a slide in the usual manner. After the blood has been mounted on it the slide is

<sup>1</sup> See Chapter XII.

incubated at  $37^{\circ}$  C. for 10 minutes, when it will be seen that the nuclei of the lymphocytes have turned scarlet.

$$\overset{\text{Lymphocytes}}{cf} = (7s + 3a + 7h + t) - (3c + n) = 14.$$

In this specimen the neutrophile leukocytes will have burst, for the jelly has an index of diffusion too high for them by 2 units—their  $cf$  being 12. For the same reason the eosinophile cells will also be achromatic, and the same may be said of the basophile cell, although it is very difficult to stain the nuclei of these cells. Their  $cf$ , however, is about the same as that of the neutrophile leucocyte.

The simple equation has other advantages. It can be inverted, so to speak, and the units of the different factors can replace each other to some extent; for since the units of the several factors are equal to one another as regards their power of increasing or decreasing the diffusion, one can interchange them at will. We can make two jellies, for instance, one of which contains 5 units of stain and 2 of alkali; and another which contains 2 units of stain and 5 of alkali; and provided the other factors are the same in the films made from each tube, the result obtained by each jelly will be identical. The equations will both give the same total:

$$cf = (5s + 2a + 4h + 2t) - (3c + n) = 9.$$

$$cf = (2s + 5a + 4h + 2t) - (3c + n) = 9.$$

Any of the factors may thus be interchanged.

We may delay this diffusion by adding more units

of salts. The  $(3c+n)$ , however, is the usual content of salts which is always present in the "coefficient jelly," but more salts may be added in the shape of solutions to the 5 cc. which also contains the stain and alkali. Whatever is added must be put down in the equation. The only substance not in the equation is agar, which, as already noted, does not affect the cells, and which is always present in the same strength in every experiment.

Since the units of the factors are equal and interchangeable, and since their sum is equivalent to the coefficient of diffusion, the numeral which constitutes the coefficient of diffusion in the equation can be interchanged with an equivalent number of units of one or more of any of the factors. We may reverse the equation, therefore, and, provided we already know the coefficient of diffusion of the cell experimented with, we can, by this reversal, determine in a moment the exact quantity of any factor required to obtain staining of the nucleus. That is to say, that if the coefficient of diffusion is known, and if all the other factors, except one, are given quantities, then we can determine the required quantity of the one unknown factor simply by reversing the equation; always remembering the well-known algebraic law that in bringing one factor from one side of the equation to the other, *we must change the signs*. For instance, suppose a strain of *Spirochæta refringens* has a coefficient of diffusion of 20, and one wishes to stain a sample of them: Let us suppose there is a jelly to hand which contains 6 units of stain, 8 units of alkali, and the usual content of salts in the "coefficient jelly"



from which it was prepared. The total contents of the tube has already been made up to 10 cc. as usual. The specimen is prepared and incubated at  $37^{\circ}$  C. Then the question must be asked, How long must the specimen remain in the incubator before the spirochætes will be stained? We could, of course, keep taking the specimen out and looking at it, but every time we did this we should lower the temperature and spoil the experiment. It is much simpler to plot the equation. The coefficient of diffusion of the spirochætes is a known quantity, *i.e.* 20; the *time* is now the unknown factor. We therefore exchange the places of the symbols *cj* and *t*, thus:

$$t = (20cj + 3c + n) - (6s + 8a + 7h) = 3.$$

$$t = 3, \text{ or } 3 \text{ units of time, } i.e. \text{ half an hour.}$$

Likewise, since the units of *all* the factors are equal, we may interchange any of them. Another example may be given. A certain strain of *Amæba coli* from a “culture” has a *cj* of 13. We want to stain the nuclei of these parasites in 10 minutes with a jelly which contains 7 units of alkali. But we want to stain them at the room temperature of  $20^{\circ}$  C. The jelly contains its usual content of sodium citrate and sodium chloride—*i.e.* 3 units of the former and 1 of the latter. How much Unna’s stain must we add to the tube of jelly to obtain the required result?

The number of units of *s* is the quantity required, hence:

$$s = (13cj + 3c + n) - (7a + 3h + t) = 6,$$

six units of stain will be required, or 0.6 cc. of Unna's dye.

The foregoing examples show how the coefficient of diffusion is determined, and how, after it has been ascertained, one can, by means of the equation, find out other factors, which may be unknown quantities. It follows that by this procedure other substances can be made to diffuse into the cells. This method of calculation has been used throughout these researches, and it will be seen that further examples will be given in the future chapters of this book.

The factors most often employed are alkali and heat. Salts are not usually varied a great deal, although their amounts can be altered if necessary by adding more of them to the second solution.

The determination of the units of any other substance is carried out on the principle that all units must be equal. Let us take a substance like urea, for instance. It delays the diffusion of other substances, such as Unna's stain. All that has to be done is to find out how much urea must be contained in the 10 cc. of jelly to neutralize the increasing action of a unit of alkali. Having found out the unit of the fresh substance, that unit is added to the equation in the usual way. If it increases diffusion it is placed in the bracket with the alkali and heat; if it delays diffusion it is bracketed with the salts.

Lastly, having obtained the coefficient of diffusion of a class of cells by measuring the rate of diffusion of the stain, the stain may be omitted and any other substance substituted for it. If more than one sub-

stance is made to diffuse into the cells, they may each affect the diffusion of the other; for they may themselves be alkalies, acids, or salts. In this case the unit of each must be found, and their action on the diffusion of other substances into the cell allowed for.

It is necessary to point out that this method is reliable only within certain limits, and precautions must be taken to prevent errors due to employing excessive amounts of substances, heat, and time, and those due to possible contingencies arising when dealing with cells from tissues.

The following list of precautions has been copied from the paper in the *Proceedings of the Royal Society*:

*Precautions.*—As regards Life and Death: In a previous paper<sup>1</sup> it has been shown that the staining of the nuclei of leucocytes, when examined by this method, is a sign of death, and that the nuclei of dead cells will stain, *ceteris paribus*, before those of living cells. Consequently all the experiments given in the present paper have been made with fresh normal cells, and in the case of micro-organisms, with cultures not more than forty-eight hours old. It may also be mentioned that the liquefaction of the cytoplasm which occurs after death materially alters the conditions of staining of leucocytes, and that the *cf* of living cells falls gradually after the blood has been shed.

As regards Excess of Alkali, causing rapid death and liquefaction of the cytoplasm, with consequent prevention of staining (achromasia): The addition of

<sup>1</sup> "On the Death of Leucocytes" (H. C. Ross, *Journal of Physiology*, vol. xxxvii., p. 327, 1908).

excess of alkali may cause death, staining of the nuclei, liquefaction, and the loss of stain on the part of the cells.<sup>1</sup> This may occur before a preparation can be focused, in which case the cells appear unstained and will refuse to stain, no matter how much stain or alkali are tried. Therefore it is better to begin with a low index of diffusion and to try tube after tube, each containing a little more alkali, until staining is obtained. Further, the amount of sodium bicarbonate should not exceed twenty units, because, as has already been pointed out in a former paper,<sup>1</sup> if added to excess it may act as a neutral salt and delay diffusion.

As regards Excess of Deficiency or Heat: A temperature above 40° C. may allow the cells to diffuse through the agar.<sup>2</sup> A temperature below 15 degrees has not been experimented with, because, even at a temperature of 20° C. it requires a minimum of 3 units of stain to cause staining of the nuclei of leucocytes in spite of the addition of a large amount of alkali, for the alkali is not sufficient, *per se*, to cause the cells to absorb sufficient stain to colour the nuclei unless the stain is concentrated.

As regards Excess of Time: A period of more than half an hour has not been employed for fear of death and liquefaction of the cytoplasm, for the cells may die and become achromatic before there has been time for sufficient stain to diffuse into them to cause staining of the nuclei, in which case, of course, the cells will never stain.

As regards Excess of Stain: More than 10 units of stain may cause precipitation of the agar as the film cools on the slide, and the precipitate carries

<sup>1</sup> "On the Death of Leucocytes" (H. C. Ross, *Journal of Physiology*, vol. xxxvii., p. 327, 1908).

<sup>2</sup> "The diffusions of Red Blood Corpuseles through Solid Nutrient Agar" (H. C. Ross, *British Medical Journal*, May 5, 1906).

some of the stain down with it, vitiating the results, for it has been shown that agar is not very soluble in cold stain.<sup>1</sup>

As regards Examination: The observation of cells floating near a bubble under the cover-glass should be avoided. The fact that blood-cells in such a situation will stain before others has already been noted.<sup>1</sup> I consider this to be due to these cells floating in a small quantity of alkaline citrated plasma collected round the bubble.

Consequently the experiments have all been made within the compass of the above restrictions. It may also be advised that when unnucleated cells contain granules in their cytoplasm the staining of the granules gives a more constant rate than the staining of the cytoplasm. By this means it is seen that the *c<sub>f</sub>* of the blood-platelet is identical with that of the polymorphonuclear cells.

<sup>1</sup> "On the Death of Leucocytes" (H. C. Ross, *Journal of Physiology*, vol. xxxvii., p. 327, 1908).

## CHAPTER VII

### DIFFUSION OF SUBSTANCE INTO CELLS TO EXCESS— DIFFUSION-VACUOLES OR “RED SPOTS”—THE PROOF THAT THE BLOOD-PLATELET IS A LIVING CELL

IN this chapter I shall discuss the effects of the diffusion of substances into a cell, when that diffusion occurs to excess. A cell's protoplasm can utilize only a certain amount of the dissolved substance or substances which diffuse into it from the immediate neighbourhood of the cell. One can, however, push this diffusion by the agency of one or both of the factors—heat and alkali—which increase diffusion, and if we do this some of the neighbouring liquid itself passes into the cell and remains suspended as minute droplets in the cytoplasm. These droplets have been called “diffusion-vacuoles.” When they were first seen, five years ago, the cells were resting on stained jelly, and since the “diffusion-vacuoles” were stained they were therefore called “red spots.”

Diffusion-vacuoles must not be confounded with the ordinary vacuoles (fig. 16) which appear as colourless, non-granular patches in leucocytes. Many theories have been advanced regarding these latter vacuoles, but although we have so often seen them, we have no explanation to offer as to their nature. They are



certainly not composed of liquid; they are not cavities; and, so far as we have observed, they play no part in cell-division. When the cytoplasm liquefies at death they disappear, and when a cell divides they seem to migrate into the cytoplasm, remaining outside the chromosomes and centrosomes.

The diffusion-vacuole is quite another kind of body (fig. 17). It is never seen in a normal cell which has been freshly removed from the tissues. "Red spots" always appear gradually (fig. 18), beginning as minute coloured points in the cytoplasm, which gradually become larger until—in the case of leucocytes—they may become as large as a lobe of the nucleus. Two or more may coalesce to form one large diffusion-vacuole; and their appearance depends entirely on the laws of diffusion; in fact, they may be produced in leucocytes at will by arranging the *plus* factors, heat and alkali, in the equation in such a way that they promote the diffusion of a substance to excess.

Diffusion-vacuoles appear only in living protoplasm. After death the cytoplasm liquefies and the cell becomes disorganized, when diffusion-vacuoles cannot appear in it. The actual passage of a substance, say, stain, through a living cell's cytoplasm occupies a certain amount of time, which can be shortened by heat or alkalies and lengthened by salts. If heat and alkali are present, but the salts are absent, the stain diffuses into the cell so quickly that death may ensue in a few moments, because the nucleus becomes stained. Indeed, one may thus cause death in a few seconds; and death is accompanied by liquefaction of the cyto-

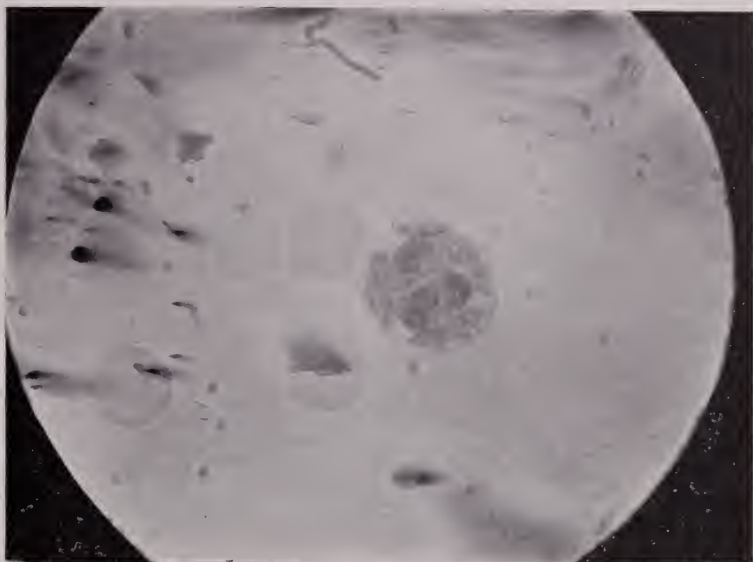


FIG. 16.—A stained leucocyte. The ordinary vacuoles (colourless patches amongst the cell-granules) are well shown. The cell has just died.



FIG. 17.—Diffusion-vacuoles in a leucocyte.



plasm, which, when it is alive, appears to be in the form of a jelly. Now, it is obvious that if the cytoplasm liquefies in a few seconds, diffusion-vacuoles cannot appear, for it is unlikely that a liquid like a solution of stain cannot remain suspended in droplets in another liquid like liquefied cytoplasm. On the other hand, if the cytoplasm is alive and jelly-like, any excess of stain which diffuses into it will become suspended in it as a "red spot." Hence, if death is caused extremely rapidly, no matter to what excess the diffusion is increased, diffusion-vacuoles will not appear, and, owing to the excess, liquid passes into the cell. If this excess is great, the dead cell will be seen to burst (it appears even to explode sometimes, especially if there are no salts to delay the diffusion), and the cell-granules are scattered about the field of the microscope. It is a well-known fact that if water is mixed with blood, the leucocytes will burst, the reason being the same, for the water passes into the killed and liquefying cytoplasm, and the intracellular tension is so great that rupture of the cell-wall takes place. There are no salts to delay the diffusion of the water, which now occurs to such excess that it causes the cell to rupture.

In order to demonstrate the diffusion-vacuoles, therefore, it is necessary to delay death, which can be done by placing salts in the jelly-film such as are present in the "coefficient jelly." Diffusion is then increased by alkali or heat until *maximum* diffusion, short of causing death, is obtained; for it must be remembered that all artificial substances will kill human cells, and the more they diffuse into them the

more rapidly will the cells die. If now half a unit more of alkali is added to the jelly, or two or three more degrees of temperature are tried, diffusion-vacuoles will gradually make their appearance in all the cells.

For instance, "red spots" are readily produced in leucocytes. Any jelly which has the correct index of diffusion for a coefficient of diffusion of 12 will cause them to appear if another drop of alkali is added to the jelly. The diffusion of the stain *must* be excessive; but not so excessive as to cause death in a few seconds. It is necessary to hit off those amounts of alkali and heat which will cause liquid to pass into the cells, but which will not unduly hasten death by staining the nucleus too rapidly. If this is done accurately, these remarkable diffusion vacuoles suddenly begin to appear. If the diffusion is still further increased, the cells will burst and become achromatic instantly. The appearance of the diffusion-vacuole may be regarded as the safety-point of diffusion; and it is a signal that no more alkali or heat may be tried, or the cells may burst.

It is interesting to watch the fate of these vacuoles. Since the substance is diffusing into the cells to excess, this diffusion must cause death in a short time, even though the cells do not burst. Before this occurs, however, the diffusion steadily increases, and the "red spots" get larger. When death takes place the cytoplasm liquefies slowly, beginning at the periphery and progressing more and more towards the nucleus. As the cytoplasm liquefies, more and



FIG. 18.—A dead leucocyte in which diffusion-vacuoles are beginning to appear.

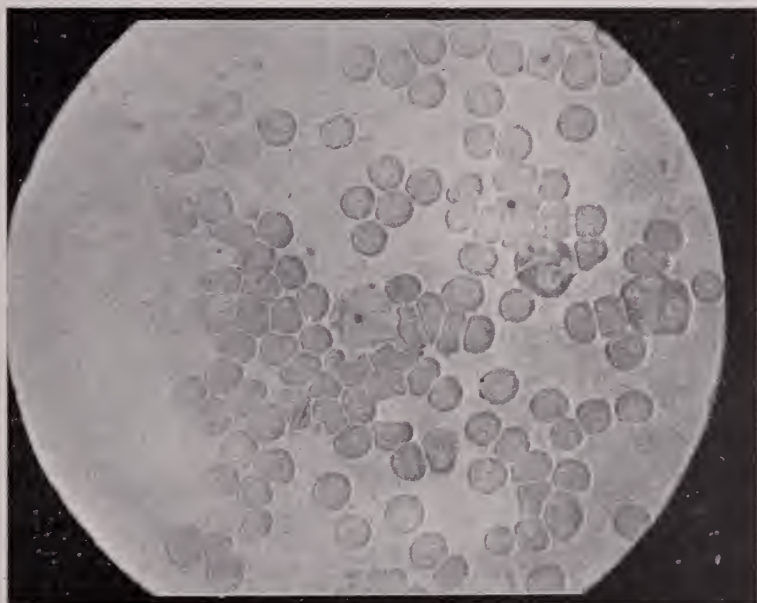


FIG. 19.—A diffusion-vacuole in a lymphocyte. Low power.





more of the cell-granules show the remarkable dancing Brownian movements, and the liquefying cytoplasm gradually involves the diffusion-vacuoles, one by one. When the liquefying cytoplasm, which immediately surrounds a vacuole, becomes of the same consistency as the liquid within the vacuole, the latter, which in reality is like a bubble of liquid suspended in a liquefying jelly, suddenly bursts and disperses, leaving a cup-shaped cavity in that portion of the more central cytoplasm which has not yet become liquefied. One by one all the vacuoles disperse, and either immediately before or after their dispersal general achromasia of the cell ensues, for achromasia also depends on the liquefaction of the cytoplasm. Vacuoles have never been seen to "disperse" in a living cell; it is necessary for the cytoplasm to liquefy for this to happen, and liquefaction occurs only at death. Diffusion-vacuoles will frequently be seen when experimenting with this *in-vitro* method, large numbers of them sometimes making their appearance in a single cell; but they will all disappear after a short time. I have seen them in all varieties of blood-cells (figs. 19, 20).

The colour of the diffusion-vacuole depends on the colour of the solution or jelly in which the cell is resting. If the jelly contains a red dye, such as polychrome blue, the vacuoles will be red; ordinary methylene blue causes them to appear blue. If no stain is present the vacuoles will be colorless; if stain is present the coloration of a vacuole is always deeper than the colour of the surrounding jelly. We

believe the reason for this is, that when the droplet of liquid becomes suspended in the jelly-like cytoplasm it forms a cavity in it, and the walls of the cavity actually become stained. This is readily seen when the vacuoles disperse, for portions of the stained wall of the cavity can be demonstrated. When cytoplasm is wounded (the formation of a vacuole in it really constitutes a wound of it) the cytoplasm stains deeply with an anilin dye, and this appears to be the reason why the "red spots" seem to be so deeply coloured. Moreover, being spherical droplets, they are highly refractile.

We have never seen diffusion-vacuoles in normal cells immediately after they have been removed from the body; it is always necessary to induce them. There is an exception to this rule, however, in the cells of some malignant growths, especially cancer of the breast, in which we have frequently seen large "red spots." We think that it is possible that the injured cytoplasm associated with these spots may produce the deeply staining patches which have been described as "archoplasm" in these cells when they are fixed and stained by the older methods. In a former paper we also suggested that archoplasm might be derived from chromatin which has diffused through the cytoplasm to some extent, and we still think that this may be possible, but it is also probable that archoplasm is derived from the fixation of injured cytoplasm connected with a diffusion-vacuole. We have never seen anything like the commonly described archoplasm in a normal living leucocyte, and it certainly does not play any rôle in their cell-division.

There appears to be little doubt that archoplasm does not exist normally in a living cell; it can be produced in them, however, by lowering their coefficient of diffusion by keeping them for some hours in extracts of dead tissues—and this is, we believe, the reason why it appears so frequently in living cancer-cells.

It is obvious that substances will, *ceteris paribus*, diffuse more readily into a cell if it has a coefficient of diffusion lower than its normal one, and, for the same reason, vacuoles can more easily be induced in it. For instance, no diffusion-vacuoles will appear in fresh leucocytes when they are resting on a jelly which will not cause the *maximum* diffusion of stain into them; but if we lower their coefficient of diffusion, and again place them on the same jelly, not only may the maximum amount of stain now pass in, but it may pass in to excess, and diffusion-vacuoles will appear. This fact has led to the determination of a point of scientific interest which has been controversial for more than half a century. It has proved that the blood-platelet is a living cell<sup>1</sup>; for diffusion-vacuoles will not appear in the normal blood-platelets, but if their coefficient of diffusion is lowered by causing gradual death, the lowering of the coefficient of diffusion so occasioned will now permit “red spots” to be induced in them.

Our experiments up to the present have revealed the fact that the coefficients of diffusion of all cells fall gradually as their vitality fails, provided this loss of vitality is gradual. The coefficient of diffusion of

<sup>1</sup>A paper by myself on “V. Ph. Vacuolation of Blood-platelets” was published in *The Proceedings of the Royal Society, B.*, vol. lxxxi, 1909.

leucocytes may fall by as much as one unit if the cells have been shed for more than twenty-four hours and kept in citrate solution at the room-temperature. There are certain substances also which expedite this loss of vitality and its accompanying lowering of the coefficient of diffusion, especially certain alkaloids and extracts of dead tissues; and it was in the course of experimentation with the alkaloid morphine hydrochloride that diffusion-vacuoles were seen in the blood-platelets for the first time.

The events which led to the discovery of diffusion-vacuoles in blood-platelets are worthy of mention.

Soon after this method of *in-vitro* staining was suggested by Professor Ronald Ross about five years ago, either he or I saw the "red spots" for the first time in leucocytes. The laws of diffusion which I have described were not then known, and only minute vacuoles had been seen in the cells, for alkalies had not been employed. These spots only appeared as minute red points in the cytoplasm, and in appearance they certainly resembled the centrosomes of plants and other cells; for it must be remembered that hitherto leucocytes have never been seen to divide, and no one knew what their centrosomes were like. In the preliminary note in *The Lancet*<sup>1</sup> by Professor Ross and Messrs. Moore and Walker, in which this *in-vitro* method was first described, these "red spots" were mentioned, and it was suggested that, from their appearance, they might possibly be centrosomes. Now, it is well known that the nature of the blood-platelet

<sup>1</sup>*The Lancet*, July 27, 1907.



FIG. 20.—A diffusion-vacuole in a granular red cell.



FIG. 21.—A clump of normal blood-platelets. They are resting on a jelly which will just stain their granules.





(fig. 21) has been a matter of great controversy for many years; some say that they are normal constituents of the blood, but are precipitates of the plasma. Others say that they are extruded nuclei of red cells, and again it has been suggested that they are derived from leucocytes. Lastly, some people say, even to this day, that they arise from all three sources. In view of this controversy, Professor Ross and his collaborators, considering the "red spots" in leucocytes to be centrosomes, suggested that if anybody could find them in the blood-platelets it would, of course, settle once and for all the real cellular nature of these bodies.

A short time after this, while I was working to determine the laws of diffusion by this method, I appreciated that "red spots" were not centrosomes at all, but were diffusion-vacuoles—a fact which I published in *The Journal of Physiology*,<sup>1</sup> and a fact which was afterwards confirmed when divisions were induced in leucocytes and the real centrosomes demonstrated.

This knowledge rendered Professor Ross's suggestion of less importance, for since the spots are *not* centrosomes, the discovery of them in the platelets would not prove that these bodies found in the blood were cells capable of reproduction. But when I was experimenting with morphia on blood-cells I accidentally discovered the "red spots" in all the blood-platelets (figs. 22, 23).

Now, in spite of the fact that these spots are not centrosomes, their appearance in the blood-platelets still proves that these minute bodies are living

<sup>1</sup> *Journal of Physiology*, vol. xxxvii, No. 4.

cells; because these diffusion-vacuoles will appear only in *living* cytoplasm.

Moreover, vacuoles will never appear in normal blood-platelets—they are never seen in fresh blood-films. It is necessary to keep the blood in an equal volume of citrated solution of morphia (a 1-per-cent solution of morphine hydrochloride in citrate solution) for four hours at 37° C. A drop of the mixture is then examined on a film of jelly in the usual way, and the film of jelly should have the correct index of diffusion, and be kept at the right temperature and time for the coefficient of diffusion of leucocytes, *i.e.* 12. The diffusion-vacuoles will then appear in all the blood-platelets. This experiment is a very easy one, and certain in its results.

The action of the morphia is the same on the blood-platelets as it is on leucocytes and lymphocytes. It lowers the coefficient of diffusion to a marked degree, and it appears to do this by causing gradual death. Morphia, in the 1-per-cent solution, is a slow poison for leucocytes, for it will kill most of them at 37° C. in about six hours. After incubation for four hours, however, when the cells are placed on the jelly, the cells are still alive, but their coefficient of diffusion is so lowered by the poison that the jelly, instead of merely causing maximum diffusion, now causes diffusion to excess, and the leucocytes and lymphocytes become intensely vacuolated (fig. 24). Further, the blood-platelets will now exhibit "red spots."

In addition to lowering the coefficient of diffusion



FIG. 22.—Diffusion-vacuoles in blood-platelets. The cells are resting on the same jelly-film as those in 21, but they had been subjected to the action of morphine hydrochloride.

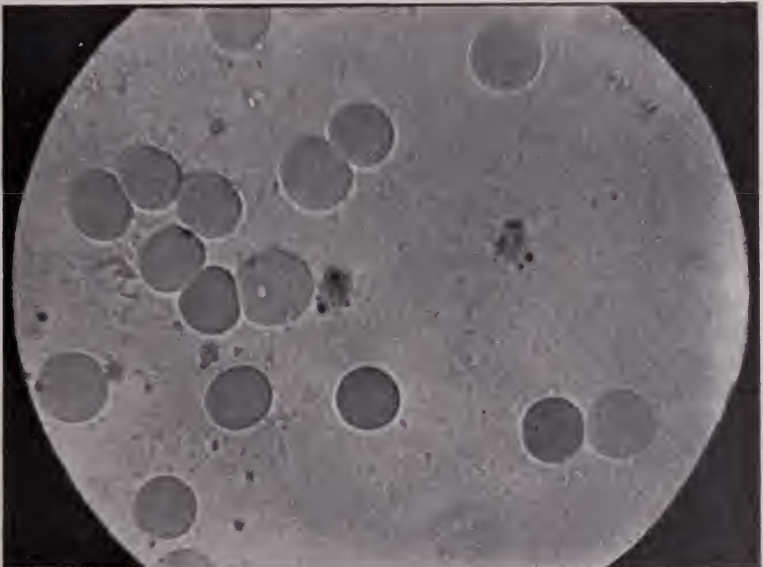


FIG. 23.—Diffusion-vacuoles in blood-platelets. The jelly-film had the same index of diffusion as that employed in 21.



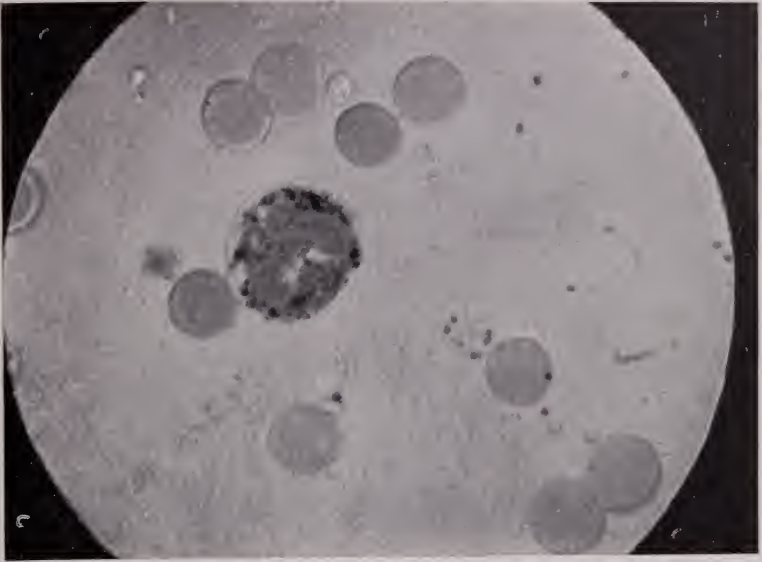


FIG. 24.—A specimen of blood which had been mixed with morphia solution. Note the extreme vacuolation of the leucocyte. A blood-platelet is also vacuolated. The same jelly as in 21.

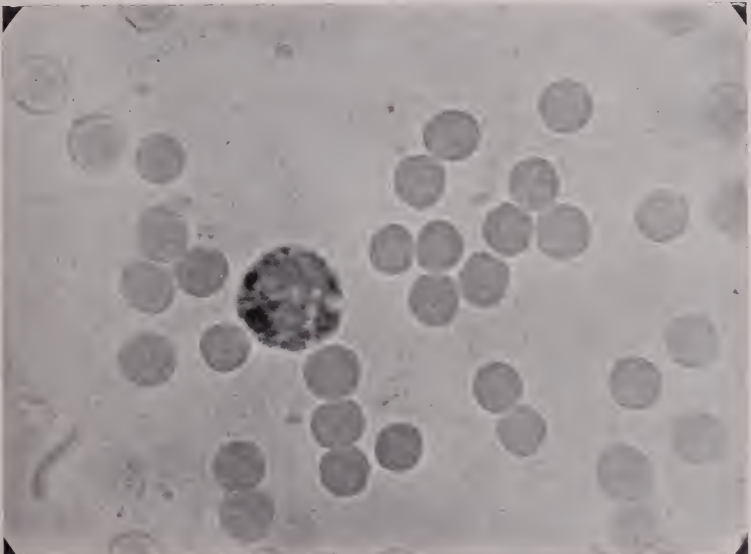


FIG. 25.—Patches resembling archoplasm induced in a leucocyte by subjecting the blood to an extract of a dead tissue. The jelly-film on which the cells are resting is similar to that employed in 21.





by causing gradual death, morphia undoubtedly has a profound effect on the cellular cytoplasm, for other alkaloids, so far as they have been tried, do not cause vacuolation of the platelets. On the other hand, we have occasionally seen a vacuolated blood-platelet from a specimen of blood which has been mixed for about twelve hours with a citrated solution (100 per cent of suprarenal extract). It has already been mentioned that extracts of dead tissues lower the coefficient of diffusion, and in producing vacuolation they also produce archoplasm in leucocytes (fig. 24). Possibly, as mentioned before, the archoplasm which is so frequently seen in cancer cells is derived from the vacuolation caused by the action of the remains of dead tissues on the cells. If leucocytes which have been subjected to morphia and have been placed on jelly as above described are watched for some time, patches which might be described as archoplasm may often be seen in them as a result of the dispersal of many of the diffusion-vacuoles induced by the alkaloid. We cannot, of course, state definitely that these patches are identical with what is known as archoplasm, and we have never seen anything which could be described as it in normal leucocytes examined by this method; but that induced in them by extracts and morphia is nearer the usual interpretation of archoplasm as seen in fixed specimens than anything we have seen.

Since the blood-platelets can be made to become vacuolated by lowering their coefficient of diffusion by the action of the poison morphia; and since all the blood-platelets in a specimen thus respond to it, it

is clear that the blood-platelet is a living cell and not a precipitate. As far as we know, no precipitate has a coefficient of diffusion, and even if such a thing were possible, one certainly could not lower it by causing approaching death with morphia.

Blood-platelets unquestionably are living cells; and they can actually be seen to be produced by leucocytes when they are examined on a jelly-film by this method. They are all the same class of cell, apparently produced in the same manner. If a specimen of fresh blood is spread on a jelly which contains stain and an alkaloid such as atropine sulphate, as will be described in the next chapter, the leucocytes and the lymphocytes become excited and extrude long pseudopodia. Sometimes these pseudopodia become detached from the cells (fig. 26), when the fragment appears to be identical with a blood-platelet. They may contain a few granules derived from the leucocytes. Moreover, the blood-platelet is also highly amœboid under this excitation; and their amœboid movements can easily be seen by this method. Deetjen, several years ago, asserted that blood-platelets showed amœboid movements, although, of course, he did not employ alkaloids to excite them. By this method, however, not only can they be readily seen to show movements, but they have also actually been photographed in the act (fig. 27). We have also succeeded in obtaining a negative of a blood-platelet apparently being produced by a leucocyte (fig. 26). As a matter of fact, the platelets stained by this method have such a remarkable resemblance to leucocytes that in the very earliest

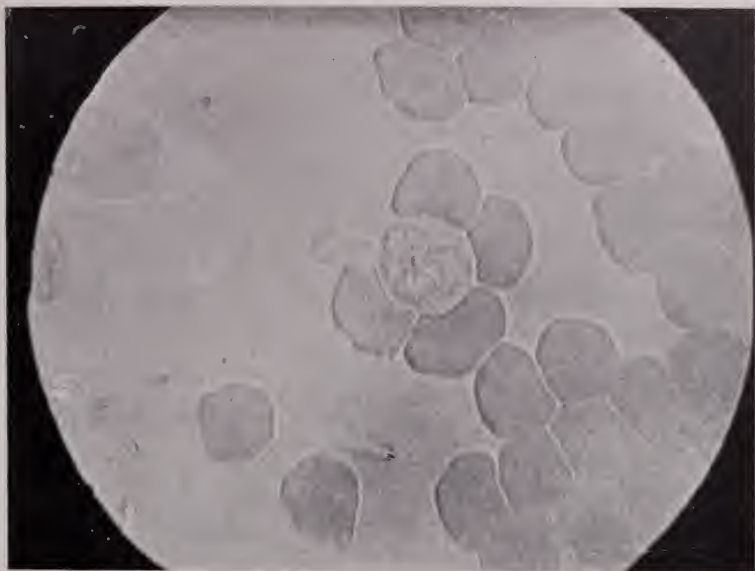


FIG. 26 — An extruded pseudopodium becoming detached from a leucocyte which is excited by atropine. No stain.



FIG. 27.—Amœboid movements excited in a blood-platelet by the action of atropine.



experiments it became apparent that these bodies were associated with those cells. We have never succeeded, however, in making a platelet reproduce itself, even with the most powerful exciter of cell-division. Blood-platelets frequently become clumped into masses, especially if the jelly contains an extract of a tissue; we therefore think that this clumping may have some function in the phenomenon of healing.

At this juncture it may be useful to dispose of an old theory, that the blood-platelet is the "extruded nucleus" of a red cell. In the first place, no diffusion-vacuoles have ever been seen within the nucleus of any cell, and the platelets, therefore, can hardly be nuclei. This suggestion of the nuclear origin of the platelet would never have occurred, I think, if the originators of it had used *in-vitro* staining. Red cells are never seen to extrude their nuclei by this method as they sometimes seem to be in the act of doing when they are spread out and fixed on a slide by the old methods. It is difficult to imagine that any cell could extrude its nucleus bodily, and a glance at the stained nucleus of an unfixed nucleated red cell will dismiss such a fallacy in very short time. The nucleus of a living red cell seems to consist merely of a mass of chromatin granules, which appear to be identical with those of the "red cell with basic granules." The granules ultimately seem to disappear altogether, for in normal blood one sees about 1 per cent. of these granular cells, which sometimes have only one or two granules, whereas in anæmia the number of granules is much greater in most of the cells. Presumably, when the granules



have disappeared altogether the cell resembles the ordinary erythrocyte. The *nucleated* red cell has a coefficient of diffusion of about 11, and so has the granular cell. An ordinary erythrocyte's coefficient of diffusion seems to be much higher, however; but since it has no nucleus or granules to stain, it is difficult to determine it.

To stain the stroma of an ordinary red cell it requires a jelly with an index of diffusion of nearly 20. Like other blood-cells, the coefficient of diffusion of red cells falls the longer the blood has been shed, until, with a jelly suitable for staining the nuclei of leucocytes, the stroma (or perhaps it is the hæmoglobin itself) of red cells will stain in many instances. It is presumed that this more rapid staining of the stroma or hæmoglobin of red cells which have been shed some time is due, as in other cells, to the lowering of the coefficient of diffusion, for extracts of dead tissues and morphia also have this effect on them.

The stroma or hæmoglobin—whichever it may be—stains more readily in nucleated and granular red cells than in the others. "Red spots" will fairly often be seen in nucleated red cells and in granular ones; but they have only been seen three times in ordinary erythrocytes.

Apart from their scientific interest, however, diffusion-vacuoles are not of great importance, we think, except that their appearance, as noted above, is a signal that maximum diffusion is being occasioned.

I have now described what we know concerning the diffusion of substance into living cells. It is a complex

subject, which will require careful elucidation if the actual physical laws on which it is based are to be found out, and I venture to think that this method will supply a means by which these laws can be determined; a large amount of careful experimentation will be necessary, however, with a large variety of substances. The chemical factors, such as alkalies and salts, will have to be tried in greater variety; after which it seems to me probable that one will be able to settle whether the increase and decrease in diffusion which they cause is due to their atomic weight or the osmotic pressure, or what. One point, however, should be clearly appreciated, which is this, that these chemical factors which increase or retard the diffusion of other substances, act not on the substance diffusing into the cell, but on the cell itself. For instance, as will be shown later on, alkalies, by increasing the diffusion of kreatin or xanthin, increase the rapidity of cell-division induced by these extractives. But the alkalies have no effect on either kreatin or xanthin. The way they increase diffusion into the cell is by causing the cell to absorb substances more readily. And so with acids, salts, and other chemical factors.

Lastly, these simple laws of diffusion must be taken into consideration throughout researches with this method, for no results will be obtained if they are forgotten. The equation has been found to be of more use when stain is employed. Later on, when one is experimenting with single substances and no stain, the arrangement of the jellies is more simple, and the equation is not used so much.

## CHAPTER VIII

### THE EXCITATION OF AMEBOID MOVEMENTS IN WHITE BLOOD-CORPUSCLES CAUSED BY ALKALOIDS

Soon after this *in-vitro* method of staining was invented, it occurred to me that it might be employed for measuring the lives of human leucocytes after their removal from the body. Much work had been done by others in the way of determining the effects of virulent disease-germs on men and animals, and soon after the discovery of "opsonins" by Wright and Douglas, many researches were made to find out how individual human cells defended the body by attacking pathogenic organisms; but little was known about the effects of virulent germs and their poisons on the protecting leucocytes. Hence, if one could measure the lives of leucocytes, it would be a simple matter to mix them with the toxins produced by bacteria, and determine the virulence of these toxins by seeing how long it took for them to kill leucocytes.

In order to measure the length of time that leucocytes will live in a given sample of blood removed from the body, it is obvious that the first thing to

be done is to be able to distinguish accurately between a living and a dead leucocyte; it is impossible to say how long a cell will live if there is no means of telling when it is dead. It may appear strange, but it is a fact, that it took two years to find out the difference in the appearance between a living and a dead leucocyte. During this two years many of the points regarding the diffusion of substances into cells, vacuolation, and achromasia were found out; but although many efforts were made experimentally to try to perfect a method of measuring the lives of leucocytes, this difficulty, that one could not accurately distinguish between living and dead cells, always stood in the way. When the point was discovered, it may almost be said that it was by accident, and even then its value as a method of measuring the lives of the cells was not appreciated for some time.

It was known, of course, that leucocytes lived for some hours after their removal from the circulation, for they sometimes showed amœboid movements; but in order to measure the lengths of their lives it was necessary to be able to say at any given moment that so many leucocytes in a given sample of blood were alive, and that so many were dead. The cells were always examined on jelly which contained stain; sometimes they showed movements and sometimes they did not; but the absence of movements was no evidence that death had taken place. Many experiments were made, and at last it was resolved deliberately to kill some cells by a virulent poison, and to see whether the cells so killed appeared in any way different from those

not so treated. The poison was mixed up with the stained jelly, and that jelly was alkaline, in order to cause the diffusion of the stain and of the poison into the cells. The poison chosen in the first instance was hydrocyanic acid, and then nitrobenzol was used; but after subjection to them, the cells presented very little difference from others not so treated and known to be alive. At last atropine sulphate was tried, with a very astonishing and unexpected result, for every leucocyte, far from being killed outright, became excited to great activity. Some time afterwards it was realised that this excitation by atropine was very constant, and that if a cell was placed on a suitable jelly which contained atropine, it would, if alive, respond with absolute certainty by exhibiting excited amœboid movements. Thus the means of measuring the lives of leucocytes was determined, and it became a simple matter, by examining the leucocytes in a given sample of blood—over a series of intervals—to discover how long they lived under varying conditions, for one was enabled at once to say whether the leucocytes were living or dead, the living ones showing exaggerated movements, the dead ones remaining immobile.

This method of measuring the lives of leucocytes, and the details connected with it, will be found in the Appendix. It was originally intended to use it for ascertaining the effect of toxins on leucocytes, and we think that for this it will have a useful application. Owing to the fact that the excitation by the alkaloid led to other work, we have not yet had time to investigate the actions of toxins very far.



Apart, however, from being able to measure the lives of leucocytes, it is very necessary in this *in-vitro* work to be able to tell at once when the cells with which we are experimenting are alive, for it is essential that one should deal only with living cells; hence we will now give the formula for the preparation of a suitable jelly which will excite amœboid movements in living leucocytes. This jelly has been called for convenience "kinetic jelly," for it will always excite living leucocytes to activity. It is as well always to have a tube of it ready to hand, in order that at any time a film may be prepared, so that we may be able to make certain that the cells in a sample of blood are alive. It is prepared as follows: To a tube of 5 cc. of "coefficient jelly" add five units (0.5 cc.) of Unna's stain, six units of alkali solution (0.6 cc. of 5-per-cent sodium bicarbonate), and 0.7 cc. of a 1-per-cent solution of atropine sulphate. The content of the tube is made up to the total of 10 cc., with 3.3 cc. of water. The mixture should be melted and boiled until it froths up in the tube, and a drop of the stained jelly poured on to a slide and allowed to set. A drop of fresh citrated blood is then placed on a cover-glass, which is inverted on to the film in the usual manner. It should be examined at the room temperature, which may be said to be about 18 or 20° C.

When the cells come to rest on the jelly they will, of course, be unstained. Slowly their granules begin to turn red. A field which contains a few leucocytes should be watched. In about fifteen minutes it will suddenly be noticed that around the circumference of



first one leucocyte and then in the others small bodies like minute beads appear. These beads seem to come from underneath the cell. The beads get larger, and quickly develop into long snake-like processes of cytoplasm, which are extruded from the cell. In a few moments every leucocyte in the specimen will apparently be putting out these long "feelers" until the cells may almost be said to look like tarantulas (fig. 28). There are usually two or three of these long pseudopodia extruded from each cell. At first the pseudopodia are composed of clear cytoplasm (fig. 29), but later on a few granules from the cell are seen to move into them. Leucocytes seem to endeavour to push their pseudopodia into the crevices between the neighbouring red cells if they can (fig. 30), although we have no reason to give for this propensity. These excited movements differ from ordinary amœboid movements in that they are far more exaggerated. The picture of a field containing several excited leucocytes is a striking one, for these movements are very different from the ordinary sluggish amœboid movements seen when the cells are merely kept on a warm stage. Moreover, it must be remembered that we are using the room temperature and no warm stage.

The excited movements are due to the action of the atropine. All the time, however, the stain is diffusing into the cells as well as the alkaloid, and as time progresses the stain will reach the nuclei which now begin to turn a faint blue colour. Now, it has already been pointed out that the staining of the nucleus of a cell

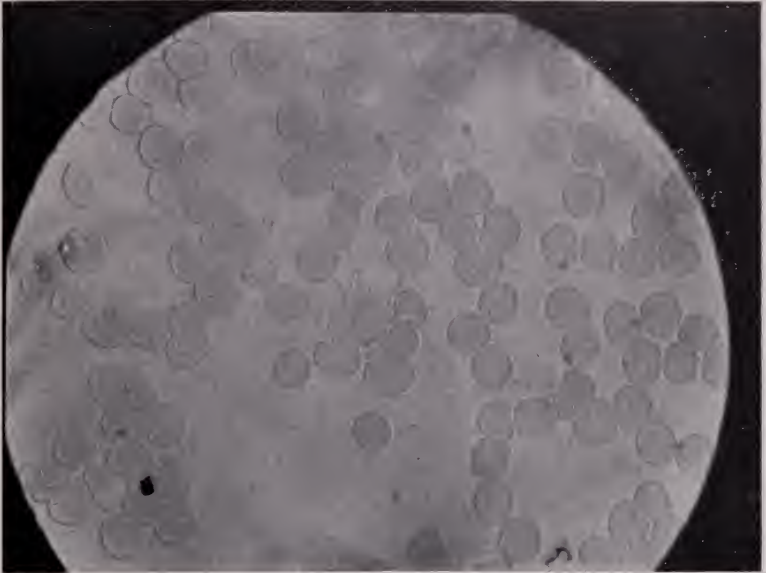


FIG. 28.—Amœboid movements excited in a leucocyte by the action of atropine. Low power.

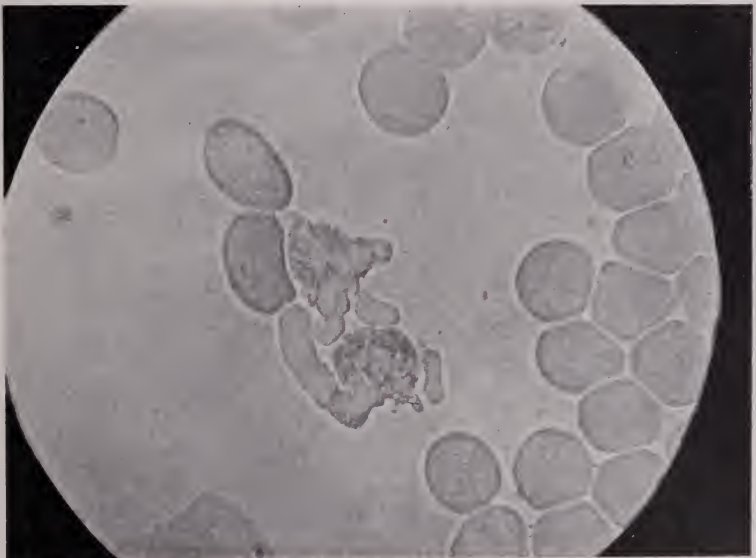


FIG. 29.—Exaggerated amœboid movements in leucocytes which have their granules stained. The movements were excited by atropine sulphate.



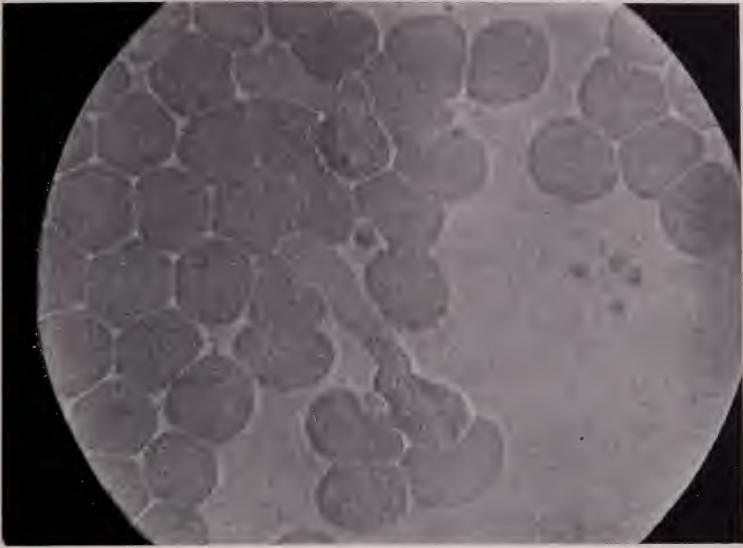


FIG. 30.—Excited leucocytes extruding their pseudopodia between red cells.

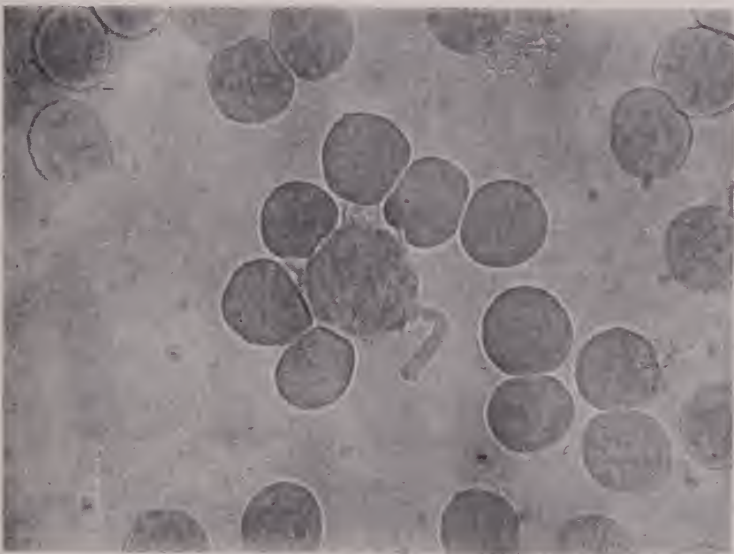


FIG. 31.—Excitation of amœboid movements in a lymphocyte by the action of atropine. No stain.



will kill it, and therefore all the leucocytes in the specimen slowly begin to retract their pseudopodia, for leucocytes endeavour to resume their spherical shape before death. The long snake-like processes can be seen to shrink back gradually into the cells (figs. 7,8), until in most cases they are completely retracted (fig. 9). Occasionally, however, a constriction appears in a pseudopodium where it arises from the cell-wall (fig. 26), and separation has actually been seen to take place; the separated portion, often containing a few cell-granules, will now resemble a blood-platelet. Soon after the pseudopodia have been retracted the cell dies, and either bursts or becomes achromatic.

If the jelly has been properly prepared the whole phenomenon of excitation of amoeboid movements will be over in about twenty-five minutes or so. The action of this kinetic jelly is instructive, for it affords another example of the diffusion of substances into the cells, and of the accuracy of the equation used in its preparation in relation to this diffusion. The way of making the jelly has been described, and it must be remembered that it contains 0.7 cc. of a 1-per-cent solution of atropine sulphate. Now, this is a salt, and it delays diffusion; hence its unit must be ascertained before the correct equation can be made for this jelly. The unit of atropine sulphate (as found by experiment) is .013 gramme, and therefore since the jelly contains 0.7 cc. of a 1-per-cent solution, it must contain 0.5 of 1 unit, which may now be added to the equation among the other salts which are *minus* factors. We may now



state the formula for the index of diffusion of this jelly:

$$fx = (5s + 6a) - (3c + n + 0.5z),$$

where  $z$  = the unit of atropine sulphate.

The specimen is kept at the room temperature, or 3 units of heat; and the object of the jelly is to excite amœboid movements in fifteen minutes (or 1.5 unit of time) in neutrophile polynuclear leucocytes, which have a  $cf$  of 12. This jelly, of course, is arranged for the coefficient of diffusion of leucocytes, and it may thus be set down:

$$cf = (5s + 6a + 3h + 1.5t) - (3c + n + 0.5z) = 11.$$

Now, if these equations are carefully considered, it should be noticed that they are apparently wrong: the coefficient of diffusion of neutrophile leucocytes is 12, not 11.

This brings us to another rule. It is obvious that if the jelly was prepared for the exact coefficient of diffusion of leucocytes, we would not obtain excitation of those cells in the given time—we would only obtain staining of their nuclei, and staining of the nuclei means that the cells would be dead. This would mean that we should defeat our object, for dead cells with their nuclei stained will certainly not respond to the atropine. “The determination of the coefficient of diffusion of nucleated cells involves death,” because the staining of the nucleus is the moment by which the  $cf$  is obtained.

But this difficulty can be overcome by subtracting

one digit from the coefficient of diffusion, and making the jelly accordingly. Hence the equation given above in reality is correct, for the coefficient of diffusion of leucocytes is 12, and subtracting one digit from it makes 11, as given in the equation. With the jelly arranged for 11, the nuclei are not yet stained, and death will not occur for another unit of time. On the other hand, the diffusion has already been sufficient for the atropine to excite the cells, and when the given fifteen minutes of time has elapsed, the cells will be seen, not dead, but in the height of their excitation.

Thus the rule is that, having ascertained the coefficient of diffusion of a cell, if we wish that cell to be alive at the expiry of the given time, subtract one digit from its coefficient of diffusion, and make the jelly accordingly.

This rule is an important one in this work, for we shall, of course, frequently have to observe cells in the act of excitation, which is an easy matter if their coefficient of diffusion is known, as it only remains to subtract one unit from any of the factors which increase diffusion, and we get the right result.<sup>1</sup>

All forms of the polynuclear leucocyte respond to atropine by exhibiting excitation of amœboid movements. In making them respond, however, the different coefficients of diffusion of each class of cell must be duly regarded. The eosinophile cell has a coefficient lower by one unit than the neutrophile; and if it is required to excite it especially, the jelly-

<sup>1</sup> It will doubtless be realised that subtracting one unit of a factor which increases diffusion, is similar in effect to subtracting one digit from the *cf.*

film must also have an index lower by one unit than that for the neutrophile corpuscle. The lymphocyte, or mononuclear corpuscle, also becomes excited to a marked degree by absorption of atropine (figs. 31-3); indeed they extrude longer pseudopodia than any of the other classes of blood-cells, a fact which is more interesting, because it is generally supposed that the lymphocyte is not a very amœboid cell, a supposition which is erroneous. To induce amœboid movements in the mononuclear cells, however, it is best to treat them as though they had a coefficient of diffusion lower than that of the leucocytes by about one unit, as these cells seem to die before the nucleus becomes stained. It was pointed out in the original specification that the staining of the nucleus indicated the point at which the coefficient of diffusion is determined. It has already been mentioned that this term nucleus is rather vague, and, as will be shown later, death is occasioned in the lymphocyte by staining of the nucleolus, which frequently becomes coloured before the nuclear wall. For general purposes, however, the original specification stands good.

The foregoing experiment, by which one can excite amœboid movements in leucocytes which have their granules stained, proves that the staining of their Altmann's granules is not very harmful to cells. The granules can, and do, become deeply stained, and all the while the cells will continue to extrude and retract pseudopodia in response to the alkaloid. This point is a very important one when we come to study induced cell-division, for it affords a clue as



FIG. 32.—Excitation of amœboid movements in a lymphocyte which has its granules stained.

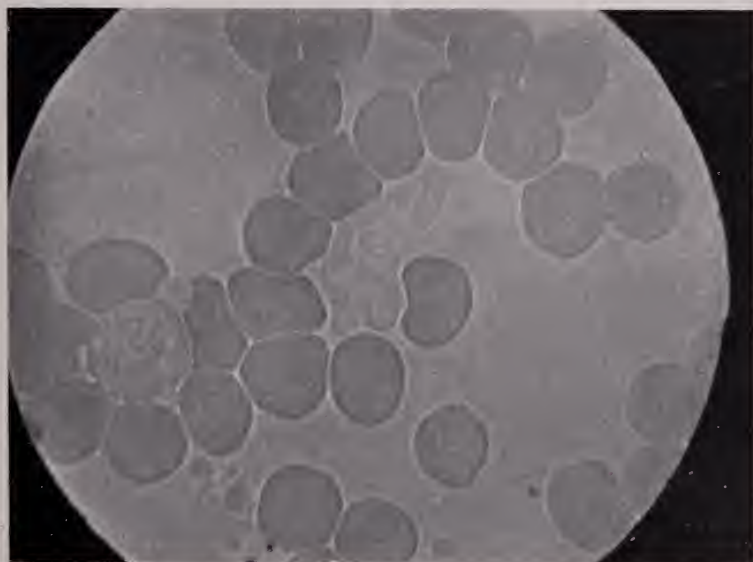


FIG. 33.—Extreme excitation of amœboid movements in a lymphocyte.  
No stain.



to how the chemical excitors of reproduction act in the causation of mitosis.

Another point is learnt, however, by experimentation with this combination of stain and atropine, for here we have two chemical agents, an anilin dye and an alkaloid, both diffusing into the cells side by side and each producing its effect on the cell-protoplasm. One excites the cell, the other kills it, and each carries out its function in direct proportion to its own concentration; for if the content of the stain in the jelly is reduced, the cells become less stained, and death is delayed; but if the alkaloid alone is reduced, the staining is as usual, but there is less excitation. At the same time, it must be remembered that the alkaloid is a salt, and, like other salts, as it diffuses itself into the cell, it delays the diffusion of the stain.

The diffusion of a combination of substances into a cell, therefore, is not a simple matter, for it represents an equation of variables, although those variables, if applied in the same manner, always have the same effect with mathematical precision.

The excitation of amœboid movements in white corpuscles is due entirely to the atropine. Using a jelly-film which is alkaline,<sup>1</sup> and which contains stain but no atropine, no amœboid movements will occur, and the cells retain their spherical shape. If the jelly is neutral, however, occasionally sluggish movements occur, even at the room temperature. At a temperature of 30 to 37° C. sluggish movements may occur

<sup>1</sup> The alkalinity of these jellies is not sufficient to precipitate the alkaloids.



even in the presence of alkali. But in any of these instances the movements are not comparable to the deliberate extrusions caused by atropine, which are very striking in character, and if once seen will always be remembered.

We can, of course, make kinetic jelly suitable for the temperature of the blood (it is merely necessary to reduce the content of alkali in the jelly by 3 units, because we increase the temperature by 3 units), and still the excitation occurs, although (and this is a remarkable circumstance) the excited movements are not so marked at the temperature of the blood as they are at that of the room. Many persons who have seen the action of kinetic jelly have disparaged it, saying that they have often seen marked amœboid movements in leucocytes; but when questioned, the fact is always elicited that they have employed the warm stage. It is the deliberate and constant exaggerated movements which invariably occur in all living leucocytes at low temperatures which constitutes the striking effect of atropine sulphate upon them. Let a control experiment be made with a jelly which contains no atropine—and no stain either if one wishes to—and the difference is immediately apparent. Excited by the alkaloid, the cells with their stained granules, extruding their long, snake-like pseudopodia in all directions, as if they were searching for something (which, as far as can be found out, they are not), form a very pretty picture, which, when seen through the microscope, will be a revelation to those who have only worked with films of dead cells.

Atropine sulphate is not the only substance which

causes this excitation. We have tried several alkaloids, and all of them have had this effect. It does not matter what the alkaloid is, nor whether it is a salt or an alkaloid; the result is the same. In fact, we think that it is probable that this power of exciting amœboid movements is a property of alkaloids generally. It is true that we have not yet tried all known alkaloids, but we have experimented with many, and we think that they probably all have this effect. Moreover, the parent substances of alkaloids, such as pyridine and quinoline, also excite white blood-corpuscles.

Some alkaloids cause more excitation than others; atropine has so far proved the most effectual, morphine the least. To man atropine is very poisonous; morphine is not so poisonous, weight for weight. To a man's leucocyte, however, it is curious to note that morphine is the more poisonous, and atropine not nearly so dangerous. By means of this jelly method we can try the effects of alkaloids and substances in various strengths on leucocytes and other cells, and if the jelly contains atropine, by noting the extent of the excitation one can find out the dose of an alkaloid which will cause maximum excitation and the dose which will cause death in a given time. Generally speaking, it requires three times as much of a given alkaloid to cause death as it does for it to cause maximum excitation.

This latter point is an important one, for it has been suggested to us that the excitation by alkaloids is in the nature of a death-struggle. It is clear, however, that if it was, the excitation would steadily

increase as more alkaloid was absorbed; but such is not the case. Moreover, this excitation is not caused by poisons, such as nitrobenzol and prussic acid. The possibility of the excitation being due to a death-struggle is also precluded by the fact that if no stain is employed the excited movements may be watched for an hour. Death-struggles, as seen in higher animals, do not usually last very long, and always commence immediately before dissolution. The excitation appears to be a specific one caused by alkaloids, although we have seen a similar form of excited movements, but not to the same extent, caused by arsenic.<sup>1</sup>

As already mentioned, we have ascertained the amounts of other alkaloids which cause maximum excitation of leucocytes, and in finding out these "doses" we have always used a similar jelly containing no stain, and the temperature employed has been that of the room in every case. The jelly was alkaline, as it contained 5 units of alkali solution, and the alkaloids were each used in a 1-per-cent solution, thus: To 5 cc. of coefficient jelly, 5 units of alkali solution, and the amount—whatever it is—of alkaloid solution were added, and the balance, up to the usual total of 10 cc., was made with water. The jelly was then boiled, and a film prepared from it in the usual way, fresh citrated blood being used in each case.

The following is a list of alkaloids which we have tried on leucocytes, and the amount of each of them

<sup>1</sup> The effects of oxygen have been tried on leucocytes by bubbling the gas through the jelly; but its action seemed to be negative.

which, when mixed with the jelly, produces maximum excitation. Treble this amount, and death will generally occur without excitation, although leucocytes will stand even ten times the dose of codeine and bruceine without dying.

To produce maximum excitation in twenty minutes:

Alkaloid.	Amount of 1-per-cent solution of it contained in 10 cc. of jelly.
Bruceine . . . . .	1 cc.
Morphine . . . . .	0.2 “
Pilocarpine Nitrate . . . . .	0.5 “
Cocaine Hydrochloride . . . . .	2 “
Strychnine . . . . .	1 “
Atrophine Sulphate . . . . .	0.7 “
Aconitine . . . . .	0.5 “
Codeine . . . . .	3 “

Atropine is undoubtedly the most active of the vegetable alkaloids; but, as will be shown later choline (figs. 34, 35), and cadaverine (fig. 36), two of the animal alkaloids produced by putrefaction, are nearly as effective. The action of morphine in exciting exaggerated movements is very poor (fig. 37), but still it does have this effect. The dose may be doubled with cocaine, and the excited movements continue. Strychnine is not so effective an excitor for leucocytes as atropine. Codeine acts more effectively than aconitine. Pyridine is fairly effective (fig. 28).

The excitation of leucocytes by alkaloids is a very remarkable thing, for it seems to be a functionless procedure on the part of the cells. The alkaloids do not appear to cause the cells to migrate at all; they remain in their original position, and merely extrude

and retract their pseudopodia aimlessly. Quinine hydrochloride excites them fairly markedly; and it must be noted that the statement has been made by other authors that quinine stops diapedesis. We have made a hanging drop preparation<sup>1</sup> with a jelly-film in such a way that the cells are not pinned down by the cover-glass, but still absorb atropine, and they therefore were in a position to move about if they wished to. They remained in their original positions, however, and seemed to be content to extrude and retract their "feelers."

Experiments were made to see if this excitation was due to any chemotactic influence of the alkaloids. Two jellies were made, one of which contained atropine and the other none; and they were so mixed on a slide that there was atropine in one part of the film and not in another. Some citrated blood was placed over the line of demarcation to see if the cells necessarily extruded their pseudopodia in the direction of the concentrated alkaloid. They did not do so, however, for, provided a cell absorbed the alkaloid sufficiently, the extrusions were made in all directions as usual.

In order to try to find out whether this excitation increased the ingestion of bacteria by leucocytes, a sample of fresh blood was mixed with a volume of citrate solution and atropine, which contained bacteria in suspension. Having been incubated for some minutes, the cells were spread on jelly; but when

<sup>1</sup> This method will be found in the Appendix, where also another method of preparing kinetic jelly will be found.



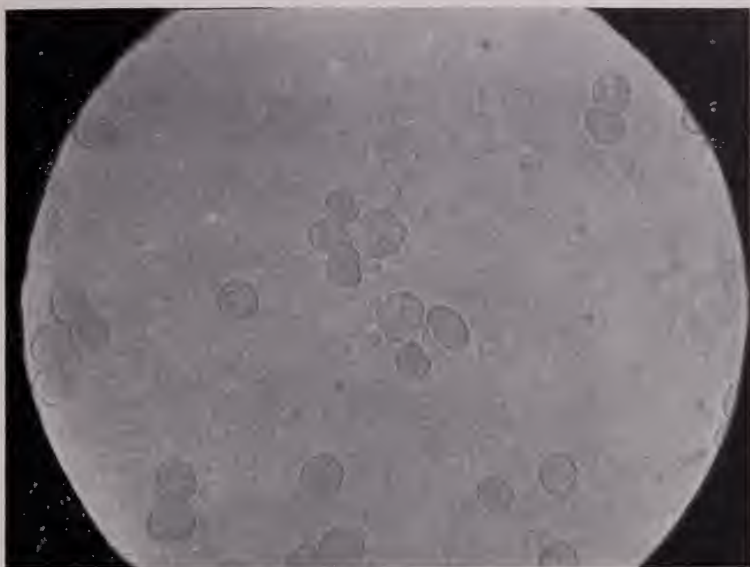


FIG. 34.—Excitation of two leucocytes by the action of choline. Low power.  
No stain.



FIG. 35.—Excitation of a lymphocyte by the action of choline. No stain.





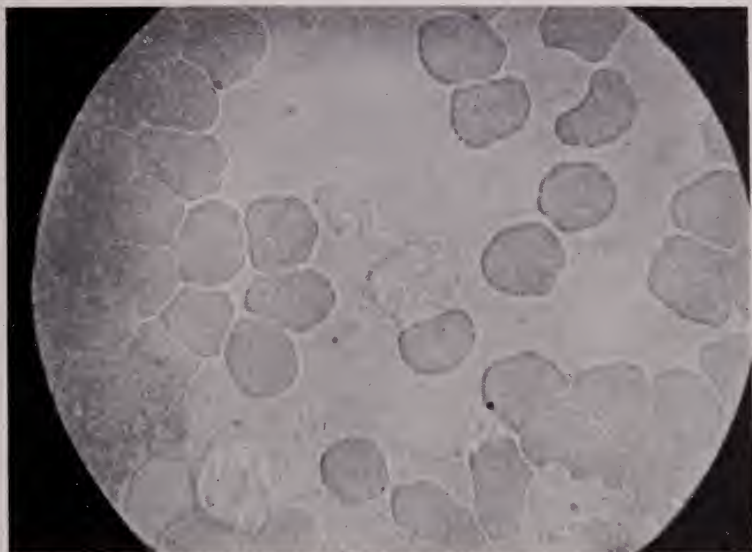


FIG. 36.—Excitation of amœboid movements in a leucocyte by the action of cadaverine. No stain.



FIG. 37.—A leucocyte excited by morphine. The cell's granules are stained.



the number of bacteria ingested were compared with those phagocytosed in control experiments where no alkaloid was used, it was seen that the excited cells did not ingest more germs than usual. Excitation, therefore, does not increase phagocytosis; and we have noticed that if a mixture of living leucocytes and germs are mixed and spread on jelly which contains atropine, the cells do not purposely extrude their pseudopodia in the direction of any bacteria which may be near them. On the contrary, if a pseudopodium happens to strike against a bacterium, the latter is usually pushed out of the way.

Whether leucocytes are excited or not, we have never seen a cell actually ingest bacteria. We have often seen cells with bacteria inside them, but we have never seen the actual act of ingestion, nor have we any explanation to offer as to how it occurs. Moreover, we have often seen leucocytes with red cells apparently inside them, although how they came to be absorbed we do not know. It is possible that the laws of diffusion may play some part in the actual act of phagocytosis.

Another point in connection with phagocytosis may be mentioned. In the making up of fixed films, germs and other substances may be crushed into leucocytes. By the examination of living cells this cannot happen. We have seen fixed specimens which showed phagocytes apparently crammed with germs; but on looking at another sample of the same cells alive a very different impression was obtained. We have mentioned this point in view of the possibility

of fallacy arising in the technique of the "opsonic index," if it is carelessly carried out, because in that technique fixed films are usually employed.

The possibility of foreign substances being crushed into cells during the preparation of fixed films is also the reason, we think, for the common, fallacious supposition—which has already been mentioned—that the blood-platelets are the extruded nuclei of red cells, for in the preparation of fixed films platelets are crushed into red cells, to which they often adhere, and after fixation they appear as if they were emerging from them; an artefact never seen with the jelly method.

In concluding this chapter it should be mentioned that Professor Osler, many years ago, pointed out that certain alkaloids excited amœboid movements in leucocytes, although this fact was not known to me when the effects of atropine mixed with the jelly-film were first tried.

As will be shown later, alkaloids have a far more important action on cells than merely exciting amœboid movements, for they greatly augment the action of the excitors of reproduction.

## CHAPTER IX

THE ADOPTION OF THE IN-VITRO METHOD FOR CANCER RESEARCH—THE EXCITATION OF LEUCOCYTES CAUSED BY CANCER PLASMA—FACTS KNOWN ABOUT CANCER—THE AGE INCIDENCE; VITALITY; DEATH; METASTASES; CHRONIC IRRITATION—THE POSSIBLE CAUSES OF CELL-PROLIFERATION DISCUSSED.

IN August, 1908, on an occasion when the excitation of leucocytes by atropine was being demonstrated to one of us (C. J. M.) he remarked that he had often enough thought that patients dying from cancer exhibited symptoms resembling those of poisoning by some alkaloids, and he suggested that an investigation might be made by means of the *in-vitro* method to try to find out whether there existed in the blood of cancer patients any substance of an alkaloidal character which might be responsible for these symptoms. This suggestion, based on bed-side observation, taken in conjunction with the fact that a group of chemical agents existed which were capable of exciting human cells, warranted a research in this direction, for if such a substance existed in the blood of these patients it was felt that either it might have



some bearing upon the cause of the disease or that it might be an effect of it.

A cancer consists essentially of cells of the body which have multiplied irregularly and too rapidly, and it was quite reasonable to think that this form of excessive proliferation might be the result of some abnormal excitation.

It should be remembered that in August, 1908, the actual *cause* of cell-division was quite unknown, and multiplication of individual human cells in direct response to a chemical agent had, of course, never been seen. It was realized that the problem of the nature of cancerous growths could only be solved by the discovery of the cause of cell-division. The cells of the body are continually multiplying by cell-division, and the correct appreciation, not only of the nature of new growths, but also of the problem of healing, and in reality most of the problems of pathology, depend upon the cause of cell-reproduction.

The most commonly accepted theory regarding the cause of cell-proliferation was that cells divided owing to some inherent vital propensity—that is to say, that they multiplied because it was their “duty” to do so. As a matter of fact, however, nothing was known as to the immediate cause of individual cell-reproduction.

So much work had been done with reference to cancer, and in spite of it so little was known concerning the cause of that disease, that we felt justified in following any clue, however slender it might appear at first sight. It was true that the excitation by alkaloids had so far only resulted in the production of exaggerated

movements in white blood-cells; but still it was an excitation, and for all we know, although they had not yet been seen, the excitation might produce other results as well. This clue, however, arising from microscopical experimentation and from a clinical observation, has proved to be of great importance, and has led by a singular chain of events to the knowledge that cell-division in the body results from the presence of specific agents, the action of which becomes remarkably augmented if the cells are in a condition of excitation resulting from the presence of an alkaloid.

This cancer research became instituted in this way, and the first step undertaken in connection with it was to test the blood of cancer patients experimentally in order to find out whether it, or other of the body fluids, contained any substance which would, like the alkaloid, excite exaggerated movements in leucocytes.

Ten cases of well-marked carcinoma were examined in the following way: A certain quantity of the patient's blood was mixed in a capillary tube with an equal volume of citrate solution. The tube was then centrifugalised and the corpuscles removed. To the remaining plasma a certain quantity of fresh blood taken from a healthy person (usually one of ourselves) was added and thoroughly mixed. The sealed tube containing the mixture was then placed in the revolving apparatus in the incubator and kept at  $37^{\circ}$  C. for half an hour, at the end of which time a drop of it was examined at  $20^{\circ}$  C. upon a slide under a cover-glass. Blood plasmata taken from fifty healthy people, or from people suffering from diseases other than cancer, were

similarly tested, and it was found that the leucocytes of healthy people bathed in the plasmata of cancer patients undoubtedly showed amœboid movements which were exaggerated and different in character from those which were observed in the corpuscles suspended in the plasmata of normal persons, or of persons suffering from a number of other diseases. The difference was one of degree, however, for leucocytes frequently under these conditions showed some amœboid movements; but we were quite satisfied that there was a distinct difference, although the test could not be considered a very delicate one.<sup>1</sup>

This series of experiments made us consider that there probably is some agent in the body fluids of cancer patients which causes excitation of cells, and one of us was charged with the task of further confirming the correctness of this observation, and of finding out what the substance is and how it is produced. It was appreciated that this substance could only be present in the blood in small concentration, and that to isolate it from serum would prove a very difficult task.

As a preliminary to this part of the research, it was considered advisable to review the known facts concerning cancer to see whether they harmonised with the possibility of the disease being associated with an excitation of cells by chemical agents. Afterwards we proceeded, by means of the new jelly method, to try the effects of different substances either taken

<sup>1</sup> A paper by Dr. Macalister and myself describing these experiments was read before the Royal Society of Medicine in November, 1908.

from growths, or which we knew were associated with growths, on individual living healthy cells. By this means it was hoped that we might find some exciting substance from cancerous growths which might in the first place cause normal individual cells to undergo a change and become similar to those cells taken from the growths themselves. In the event of such a substance being found, it would, of course, then be necessary to try to prove the argument by experimentation with the substance in the body itself. In other words: believing that cancer might be due to a chemical agent, we proposed to try to find that agent, and to test its effect, in the first instance on individual cells under the microscope, and lastly to test its action on groups of cells in the tissues of the body.

Malignant disease may be separated into two main divisions—carcinoma and sarcoma. The former attacks gland-tissues and epithelial cells; the latter is a disease of connective tissues. These researches are almost entirely concerned with carcinoma, and the term “cancer” in this book refers to that disease. There is, we think, a close association between these two forms of malignant disease, although there is a line of demarcation in the age incidence and in some of their morphological and clinical characteristics which separates them. Cancer—that is to say, carcinoma—attacks people over the age of forty, although there are occasional exceptions to this rule; but sarcoma may occur at any age from infancy onwards. At the outset we turned our attention exclusively to the

consideration of carcinoma, for we considered that if we succeeded in throwing any light on the causation of that disease, it would be time enough for us to investigate sarcoma. Cancer is much more common than sarcoma; but it has to be remembered throughout that, from the similarity of the cardinal symptoms of the two diseases, there is probably an intimate association between the causes of both. The connective tissues can become malignant at any age. The epithelial tissues are usually attacked after the age of forty. This age-incidence of carcinoma is most striking, and it necessarily constitutes a fundamental fact with which all our thoughts regarding the cause of cancer must ultimately harmonize. It is a disease of senescence; it attacks people when they are robust and apparently in a state of highest vitality, just when they are in the prime of life, or having just passed it. We have to remember in this connection that the expression "prime of life" in its physiological sense may be taken to refer to middle life—that is, somewhere about the age of 35; and we may further understand that before that age a man is being built up, whereas afterwards he enters upon the downward trend and steadily progresses towards physiological death, which may be taken to occur about the seventieth year. We may therefore consider that the climax of his physiological life is reached at 35.

The age incidence of cancer is unique; there is no other disease which has this limitation in its age averages. Exceptions do occur, it is true, but the number of cases occurring during senescence, when



man has passed the climax of his age, is so enormous that the possibility of fallacy due to "the error of random sampling" is reduced almost to zero. It is a salient feature of the disease which cannot be disputed, and we may regard it as an axiom that cancer attacks people when they are trending downwards from their physiological prime. The question is, therefore, What happens in the tissues during this senescence which renders them liable to the onset of cancer? At the time when these researches were first applied to the investigation of cancer, this question could only be answered in a speculative manner; but it was appreciated that the conditions present after the prime of life which predisposed to the disease might merely depend on something in the nature of the oversetting of a physiological balance.

Vitality seems to be worthy of consideration as a factor connected with the onset of malignancy. Very old persons do not appear to be so liable to cancer as those between the ages of 40 and 55—a circumstance which may possibly be due to a loss of vitality, for it has already been mentioned that cancer is a disease of the robust. Premature ageing, on the other hand, seems to favour the onset of cancer; but in conditions of decrepitude there is more freedom from it. Tottering persons, such as are seen in asylums and institutions, do not so frequently develop carcinoma; but people who are sufferers during their senescence from the atrophic form of osteo-arthritis or from gout are common victims. Let the reader visit a home for incurables, and he will there learn that many of the



cases of cancer arising in the institution are also afflicted with rheumatoid arthritis. The ætiology of cancer is a large subject, and for full information regarding what is known of it reference may be made to an excellent volume by Mr. W. T. Gibson, on *The Etiology and Nature of Cancerous and other Growths*.

This book enumerates in detail the trades and professions the members of which are especially prone to cancer, and it furnishes a valuable aid to pathological cancer research. Therein it is shown that chronic alcoholism is a predisposing factor. Syphilis also is undoubtedly a predisposing cause of cancer, provided the disease is not too severe. We have been reminded of this point by Mr. Pernet,<sup>1</sup> whose experience of syphilitic patients has left him convinced of an association between the two diseases.

The conditions of decrepitude and chronic enfeeblement—to which reference has been made as ones which render persons less liable to malignancy—affect not only the general vitality of the body, but also presumably the vitality of the individual cells.

Cancer is a disease which is general throughout the world as far as we can find out, but climatic conditions appear to influence its incidence to some extent. Sir William MacGregor has told one of us that as far as he can remember he has never seen a case among the Esquimos, an observation which is interesting in connection with the association of cancer and some putrefactive products, which will be discussed in the

<sup>1</sup> "The Intramuscular Syphilitic Treatment," by George Pernet, *Transactions of the American Medical Association*, June, 1909.

later chapters of this book, for, generally speaking, putrefaction of organic substances must be reduced to a minimum in the ice-bound regions of the far North.

Death is the ultimate result of cancer in most instances, unless the progress of the disease is successfully interrupted by surgery, and this is a fact which must be carefully considered. Cancer consists of a growth, of human cells. Why should such a growth kill the person it afflicts. Benign growths do not necessarily cause death? It may be, of course, that the original cause of the disease increases with the growth, and that it is this cause which is instrumental in killing the victim. We speak of death from cancer as resulting from the vague conditions described as "exhaustion and cachexia," but why these conditions result from cancer was not even within the realms of speculation.

Cancer is a disease which seems to aggravate itself. Once the disease is started in the circumscribed area—for it always begins in one spot—it will go on steadily if it is left to itself. Moreover, one of the features of a malignant growth is that it produces metastases. Why should malignant growths and not benign ones produce metastases? It is usually considered that metastases are due to embolism, and that the transplanted cells continue to multiply in their new surroundings; but, again, why should these emboli only continue to multiply in *malignant* tumours? Benign growths, like the malignant ones, are supplied with vessels and lymphatics, and there seems to be just as much reason why portions of both forms of growth should be swept away to form

metastases in other parts of the body. Still, the fact remains that metastases occur only in malignant disease.

The foregoing points formed our axioms. Whatever experiments we undertook had to harmonise with them all in their consideration. There was one other factor, however, which has already been mentioned; the mystery of the cause of cell-division in the body, and a well-known predisposing factor in the causation of cancer which is intimately associated with it, namely, *chronic irritation*.

The body consists almost entirely of living cells; individual living creatures, each of which is capable of separate existence for a short time, but which in conjunction with one another form the tissues which in their turn have special functions. Each cell is merely an individual in a multitude; a unit in an organ. Cells not only have functions to perform for their own individual welfare, but they also act collectively for the general welfare of the body.

Since cancer consists of a tumour composed of cells, we may attack the problem of its causation from two aspects—the investigation of the individual cells, and the investigation of collective masses of them. At the outset, the first aspect is obviously the one to receive consideration; and since cancer consists of a growth of cells which have multiplied too often and have so formed a tumour, the first question to be asked is, What makes this excessive multiplication take place?

Before this question can be approached, however, another question must be answered, namely, What makes *any* multiplication of cells take place?

The multitudes of cells which form our bodies have been evolved from a single pair of cells. The maternal ovum is a single cell, and always remains as such until it is fertilised by the paternal spermatozoon, which in its turn is also a single cell. The conjugation of the two at once causes cell-division to take place within the egg. Multiplication occurs, and where there was one cell there are now two; and each daughter cell divides and divides until generation after generation of new cells are produced, and this cell-reproduction ultimately leads to the formation of the new individual. The basis of the formation of new beings is the reproduction of cells by their division in response to the conjugation of an original pair of cells. We had therefore to ask ourselves why this conjugation should cause cell-division; but unfortunately the answer was unknown.

Throughout our lives, although we cannot actually feel it, the cells in our bodies are continually reproducing themselves by division by mitosis, and individual cell-death is also constantly taking place. It is true that some cells, such as some cells of the nervous system, probably live throughout the length of our lives, but myriads of other cells are constantly dividing to help to build up the tissues. "Birth" and death are continually going on among the individual units of ourselves. When a tissue is sectioned and examined microscopically it will frequently be seen that some of the cells are in the act of division by mitosis; but when we asked what makes the division occur, and what makes cells multiply to build up the tissues, we could only say once more that the reason was quite unknown.

When a child grows to form a man he grows by the multiplication of his cells, but we did not know what causes him to grow, or what makes his cells to attain this object.

Again, if we injure or wound ourselves in any part of the body, the tissues always make an attempt to repair the damage. No matter to what extent the injury may occur, attempted healing always takes place. The phenomenon of healing is due to the proliferation of white blood-cells, which multiply by cell-division to repair the tissues which are damaged. Not only do leucocytes and lymphocytes proliferate when a tissue is damaged, but other cells also multiply. For instance, epithelial cells will also proliferate to heal a damaged site. The cell-proliferation of healing forms one of the bases of pathology, and therefore of medicine also; yet it had to be admitted that nothing whatever was known as to why this cell-proliferation occurs when any part of our bodies is damaged. The process of healing is occurring in our bodies throughout our lives, and this sudden multiplication of cells must be constantly before the consideration of medical men; but although this multiplication by reproduction is an established fact, one never hears the question asked, Why do cells immediately divide to reproduce themselves when a tissue is damaged? If the question was asked, however, the answer would have had to be, "We do not know."

For the cell-proliferation of healing to occur it is not necessary for the skin to be actually broken. On the contrary, extensive cell-proliferation of healing may



occur as the result of a bruise or disease; and chronic irritation, which is an invariable predisposing factor in cancer, may give rise to exuberant multiplication of the cells in the neighbourhood of the irritated part. A common instance of the proliferation due to chronic irritation is shown in the case of a "corn." An ill-fitting boot irritates a certain portion of the foot by pressing unduly on a certain portion of the skin. The skin becomes hardened, and a small tumour may even be formed on the irritated spot. This hardening is due to excessive proliferation of the living cells in and immediately underneath the skin. A wart is an example of the proliferation due to irritation; but although this irritation leads to proliferation, we do not know exactly why the cells proliferate in response to it. If we think the problem out carefully, we can picture a group of living cells multiplying by division, and then try to grasp how irritation of that group by mechanical pressure can possibly make the individual cells reproduce themselves; for this is what they do. At the time when these cancer researches were started we could not grasp this point. It seemed incredible that a cell would reproduce itself because its cell-wall was "tickled" or pressed upon.<sup>1</sup> Why should a living cell undergo the complex phenomenon of mitosis for a reason of this nature? Besides, living cells are very delicate, and we know that they will not stand much handling or pressure without dying. No; it was necessary to find some better explanation of the cause of cell-division than mere mechanical irritation, and we appreciated that irritation

<sup>1</sup> The pressure of a cover-glass does not cause cell-division.



in reality causes localized cell-death, and the cause of the proliferation due to irritation would in all probability be found to be due to the same cause or causes which make our cells multiply in order to heal up a cut or a sore.

Now, there can be no doubt that cancer occurs in sites where there has been previously some form of chronic irritation, and cancer is another name for malignant proliferation of cells. Since such irritation is probably directly associated with the cause of the cell-proliferation of healing, we made our first endeavours to try to find out this cause of cell-reproduction in the body, for it was considered that if that could be found a step in the right direction would be made. Some attempts at healing are always going on in the parts that are subject to chronic irritation, and we may safely say that the cell-proliferation of healing is going on in these parts. Cancer supervenes on old ulcers and sores, which, of course, are healing sites. In the breast and uterus, two of the commonest places for cancer, the cell-proliferation of healing occurs every month during the ages of actual sexual function, and at the climacteric a large involution takes place, accompanied by destruction of tissue. The irritation which causes cancer of the lip is usually the pressure of a tobacco-pipe; X-ray cancer usually follows the ulceration and damage due to burns; and there are many other examples. The cause of the cell-proliferation of healing, therefore, constituted our first investigation in the path of cancer research. Leucocytes and lymphocytes are the cells which pro-

liferate to a great extent when healing occurs anywhere, and these white blood-corpuscles formed the objects of our observation in the first instance. What made these cells divide we did not know, how they divided was also unknown; but we knew that amœboid movements could be excited in them by means of alkaloids.

It is an astonishing thing that when any injury occurs, in no matter what part of the body, those neighbouring cells which have not been damaged will immediately reproduce themselves. If the damage is persistent, and healing becomes very chronic in persons over the age of 40, cells may reproduce themselves in a malignant manner, and then they go on dividing and dividing, producing a cancerous growth which ultimately kills the person the part of whose body they are, and whose damaged tissue it was their endeavour to heal. The first thing to do, undoubtedly, was to try to find the cause of the cell-proliferation of healing.

## CHAPTER X

EXPERIMENTS WITH NUCLEIN—THE LOWERING OF THE  
COEFFICIENT OF DIFFUSION CAUSED BY EXTRACTS  
OF DEAD HÆMAL GLAND—DIVISIONS INDUCED IN  
LYMPHOCYTES FOR THE FIRST TIME—REVELATIONS  
CONCERNING THESE DIVISIONS—THE RÔLES PLAYED  
BY THE ALTMANN'S GRANULES, NUCLEI, AND  
NUCLEOLI IN THEIR CELL-DIVISION

BEFORE proceeding to discuss the problem of the causation of cell-division, it is necessary to state that another piece of information was at our disposal which we believed to be intimately associated with cell-division, although the fact was not appreciated when the point was first noticed. During experimentation with a mixture of stain and alkaloid on blood-cells it was noticed that with a citrated mixture of Unna's stain and the alkaloid atropine the lymphocytes sometimes extrude granules (fig. 39) from their cell-walls. These granules remain attached to the cell by means of a streamer, apparently derived from the cell-wall itself. The extrusion appears to be a deliberate one on the part of the cell, and the granule ultimately becomes separated from it altogether. This extrusion, or "flagellation" as we erroneously called it, has been confirmed

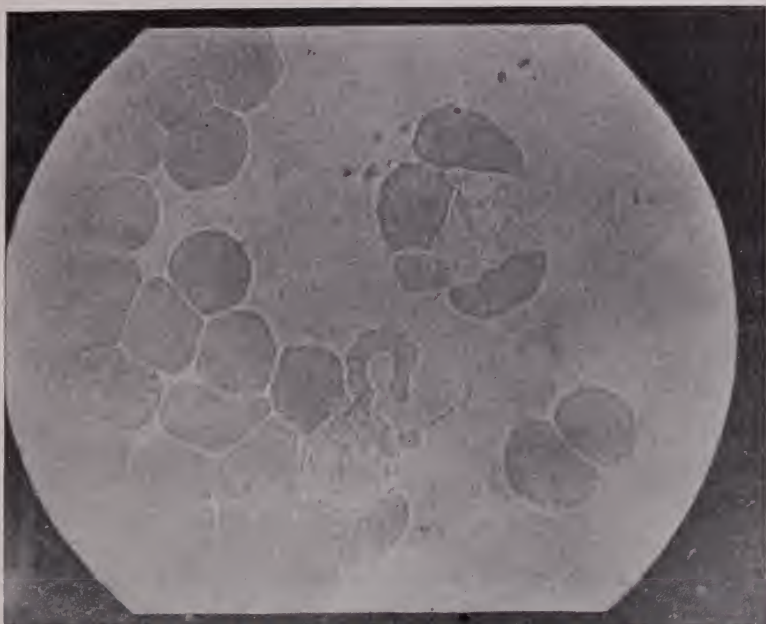


FIG. 38.—Leucocytes excited by pyridine. No stain.

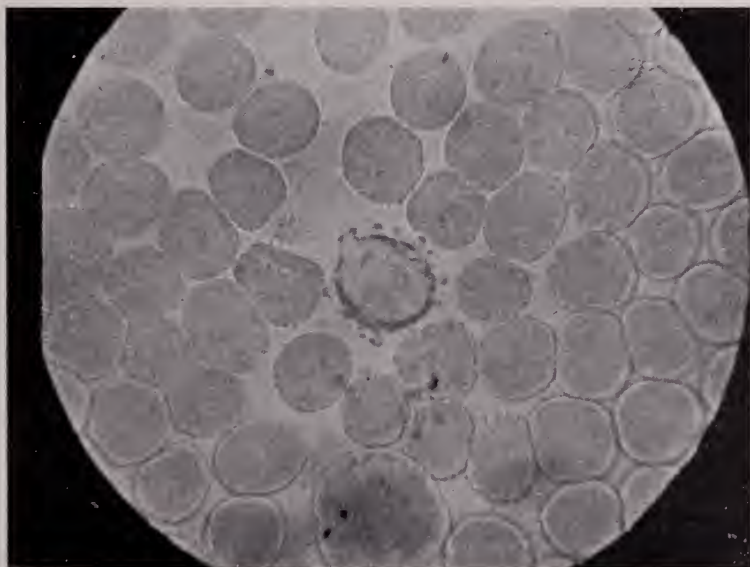


FIG. 39.—A lymphocyte which has absorbed stain and atropine discarding its granules (flagellation).



by L'Engle, of Philadelphia, who has also seen it occur in polynuclear leucocytes. When we examined fresh blood-cells mixed with the plasma of cancer patients, we again noticed that the lymphocytes extruded granules in some cases apparently in response to something in the cancer plasma, a point which Dr. Macalister and I published in *The British Medical Journal* on January 16, 1909. Dr. Buchanan, however, has informed us—and this is a most interesting point—that he had previously seen similar extrusions take place in cases of leukæmia, a fact which he mentions in his book; and a fact which we shall recall later on. We, however, had never seen these extrusions occur unless alkaloid or cancer plasma had been mixed with the cells.

As already mentioned, the commonly accepted explanation regarding the cause of the reproduction of cells by individual cell-division is not very satisfactory. One of the characteristics of living matter is that it is capable of reproducing itself, and the theory as to its causation in animal cells was that they, being living creatures, reproduce themselves because it is an intrinsic function of the protoplasm—that is to say, that it is a vital propensity on the part of every cell to divide automatically, so to speak, and to continue to do so until it dies. This explanation, however, does not harmonise with certain known facts concerning cell-proliferation. For instance, physiologically cell-division is influenced by conditions outside the cell. The limitation of the size of an organ must be controlled by some governing factor which influences not only the



proliferation of individual cells, but that of multitudes of them. It is very difficult to believe that the development of an animal from the ovum can be entirely an automatic function of the protoplasm of individual cells, unless that function is so controlled that the cells act together in masses. Moreover, the phenomenon of healing which has been mentioned presents features which tend to dispose of the "automatic theory"—a theory which does not explain why cells immediately reproduce themselves at a much quicker rate than normally when a tissue is damaged. Leucocytes, for instance, will not divide when they are removed from the body, nor have they ever been seen in the act of division when examined from the peripheral circulation. Yet when these cells are shed into a damaged tissue they proliferate immediately.

Jacques Loeb was, we believe, the first to show that cell-division in the ova of star-fish can be accelerated by certain chemical reagents; and further observations were made in this line of work by B. Moore, H. E. Roaf, and E. Whitley, who proved that the regularity and rapidity of growth of the cells of the fertilised ova of echinoderms could be greatly influenced by certain alterations in the alkalinity of the water in which they normally divide. B. Moore has also shown that the alkalinity of the blood-plasma in cancer is increased—a point which is of great importance, especially when we remember that alkalies increase the diffusion of substances into living cells.

O. and R. Hertwig and Galleoti have described how, when mitosis occurs in some of the cells of

lower animals which have been subjected to certain alkaloids, such as quinine, nicotine, and cocaine, and also to antipyrine, the mitotic figures may be of the asymmetrical type, and that in the case of certain epithelial cells of salamanders the mitotic divisions which occur in the presence of these substances closely resemble the asymmetrical divisions seen in human cancer-cells. These points took us back once more to our own knowledge that alkaloids excited leucocytes; but we have never seen divisions, asymmetrical or otherwise, actually induced in leucocytes by any alkaloid or other substance.

Farmer, Moore, and Walker had closely studied the cytology of cancer-cells. They had frequently seen cells in the act of division in their stained specimens, and they described the asymmetrical "maiotic" mitoses by which cancer-cells frequently appear to divide. By the expression "maiotic division" a "reduced" division is meant; that is to say, that a cell divides with a reduced number of chromosomes, and instead of having its customary somatic number, that number may be reduced to one-half. In man the somatic number of chromosomes is thirty-two, and cancer-cells sometimes divide with sixteen chromosomes. Farmer, Moore, and Walker also describe other characteristics of the several maiotic phases of mitosis, and they specify two forms of maiotic division—namely, the first change in a cell's life-history from its somatic division to the maiotic, which they call the first (heterotype) maiotic division, and the succeeding maiotic divisions of its life-history, which

are called the homotype maiotic divisions. These authors, however, believe that it is not only cancer-cells which divide by maiotic divisions, but that certain other tissue-cells also normally proliferate by maiotic reproduction, especially some cells of the testis, and the "wandering" cells of the body.

In March, 1909 we discussed the problem of the causation of cell-division with Professor Harvey Gibson, who suggested that we might try the effect of nuclein on cells; and he founded this idea on the well-known fact that in the sexual generation of the normal alternations of generations of plants the nuclei have only half the number of chromosomes which are present in the nuclei of the asexual generation, and that what is normal in the plant appears to resemble what is pathological in the human being's cancer-cells. It is thus suggestive that a cancerous growth might be looked upon as consisting of abnormally induced "gametophytic" or sexual tissue. Professor Gibson, with this in his mind, suggested that it might be possible by some means to induce the nuclei deficient in nuclein to absorb more, and so get back to the normal somatic condition. Farmer and others have shown that it is possible to induce such changes in the tissue of ferns, and for many months one of us (C. J. M.), acting on this knowledge, treated some cancer patients with nuclein, which was made by Professor Reynolds Green, but without proof that it conferred benefit. We, however, determined to experiment with it on individual cells.<sup>1</sup>

<sup>1</sup> Quoted from a paper, "A Report on Cancer Research," by Dr. Macalister and myself, in *The British Medical Journal*, October 23, 1909.

From the foregoing facts, believing that it was reasonable to suppose that chemical agents might influence human cell-division, we resolved to try the new *in-vitro* method. Bearing in mind that the cell-proliferation of healing appeared to be associated with the proliferation of cancer, our first step was to try the effect of nuclein on leucocytes. A saturated solution of it was made in "citrate solution," and this was mixed with an equal volume of fresh blood. It was found that the nuclein seemed to lower the coefficient of diffusion of the cells very markedly compared with a control experiment in which no nuclein was employed. Some nuclein was then mixed up with jelly which contained stain and which had the right index of diffusion to stain leucocytes deeply, without killing them, in twenty minutes. But nuclein did not excite amœboid movements in the cells.

In the next place some juice was squeezed from a malignant growth and citrated, and the citrated mixture was in its turn mixed with some fresh normal blood. It was found that this juice, like the nuclein, lowered the coefficient of diffusion of the leucocytes, but in addition it excited amœboid movements in them.

The lowering of the coefficient of diffusion due to nuclein was striking, because not only does the juice of a growth do the same thing, but the cells of cancer patients usually have a lowered coefficient.

We were not satisfied, however, with this experiment with nuclein, because the preparation of it which we had obtained was very insoluble in neutral solution, and it was impossible to employ it in any more concen-

trated form because more powerful solvents damaged or killed the cells. In place of this nuclein, therefore, extracts of some dead tissues were made, which we believed would contain the dead chromatin of cells, and it is said that chromatin contains nuclein. Moreover, it was thought advisable to keep as closely as possible to chemical substances which might be produced in the body, and the insoluble nuclein which we had used had been extracted by an elaborate process with hydrochloric acid.

To obtain this extract containing—as we believed—the chromatin of cells, we adopted a principle based on our observations of the phenomenon of achromasia. It may be recalled that achromasia is believed to be due to the chromatin of cells passing out of them, by dialysis, after their death. Achromasia will readily occur if cells are allowed to die in a solution which contains salt; and its onset after death is accelerated by heat. We therefore made an extract of a tissue by chopping it up in “citrate solution” and keeping it for twenty-four hours at 60° C. The first tissue chosen was lymphatic gland—the reasons for this being the knowledge that cancer frequently spreads through the lymphatic channels and glands, that lymphocytes are always seen in large numbers in growths, that lymphatic glands contain large numbers of lymphocytes, and especially because lymphocytes proliferate to a large extent when a tissue is chronically damaged.

The small prevertebral (hæmal) glands of lambs provided the lymphocytes whose chromatin we hoped to extract. These glands are composed almost entirely



of lymphocytes. In the first instance a dilute extract was made in citrate solution, kept at 60° C. for twenty-four hours, and then filtered. Some fresh blood was mixed in a capillary tube with an equal volume of the filtrate and incubated at 37° C. for three hours. A drop of the mixture was then examined on the stained jelly which excites amœboid movement in leucocytes (kinetic jelly). It was at once seen that the coefficient of diffusion of the leucocytes and lymphocytes had fallen remarkably—a greater fall than had ever been seen except that produced by morphine. The nuclei actually stained on this jelly in about fifteen minutes; and this jelly will never stain the nuclei of normal leucocytes—for they burst before that happens. It was also noticed, however, that the cells contained oval vesicles within their cytoplasm which closely resembled “Plimmer’s bodies.” After a while these bodies became identical with diffusion-vacuoles of large size, and when they burst some of them resembled archoplasm. It may be noted that other authors have suggested that “Plimmer’s bodies” and archoplasm are identical. We think that these vesicles induced in leucocytes by the extract are diffusion-vacuoles due to the lowered coefficient of diffusion.

The next series of experiments was made to observe the effects of this extract of hæmal gland on leucocytes when the cells are spread on jelly which contains the extract. The jelly-films also contained the correct amount of Unna’s stain to stain the granules of the cells, so that, if the extract had any action on the individual cells under these conditions, they would be



observed nicely stained and yet alive while this action was taking place. At first a dilute extract was used, as before, and the films in some instances were incubated for a short time, while others were suitably prepared for the room temperature. In one or two cases the lymphocytes seemed to contain some rod-shaped bodies in the cytoplasm. These rods stained a bright scarlet, similar to the staining of chromatin, and nothing had been seen like them before. They certainly were not bacteria, for we have often seen ingested bacteria which have quite a different appearance; besides, they were only seen in the lymphocytes, which we have never seen to ingest bacteria. With great hesitation we thought they might be chromosomes.

Before proceeding farther it is necessary to explain that at the time when these experiments were made the appearance presented by the chromosomes of lymphocytes were unknown; in fact, it was not known whether these cells from the peripheral circulation divided by true mitosis or not. One of us had examined leucocytes by the *in-vitro* method for four years, and had never seen anything, previous to these last experiments, which appeared in any way connected with division of the white blood-cells. It was appreciated that, with the new method, a possibility existed that cell-division in white blood-cells might some day be seen; but to observe what appeared to be chromosomes in lymphocytes, after we had tried only one or two groups of substances, seemed to be too good to be realised. It was necessary to be very careful, however,

before we came to any conclusion as to the nature of the bodies which had been observed in the cells.

The first striking point noticed about the red-staining rods was that they were not within the nuclei, but were in the cytoplasm outside the nucleus. This did not seem to be right, if the rods were chromosomes. It is usually considered that the phenomena of mitosis goes on within the nucleus as it does in plant-cells. Hitherto, mitosis in human cells, or animal cells generally, had been seen only in cells which had been killed and fixed with heat or chemical agents at a time when they happened to be in the act of cell-division. From observation by the older method, it was usually understood that during mitosis the nuclear wall vanishes, and the chromatin within the nucleus forms into chromosomes, which then migrate into the cytoplasm. We were prepared to believe that the older methods might be fallacious owing to distortion caused by the killing and fixing of the cells, and to the fact that cells were only caught in the act of mitosis, not observed undergoing the whole phenomenon from start to finish. If our observations were correct, the rods in the lymphocytes were in the cytoplasm right enough, but the nuclear wall was still there *internal to* the chromosomes.

The experimentation was then improved. Instead of the dilute extract being used, a concentrated one was made consisting of 50 grammes of hæmal gland chopped up in 50 cc. (*i.e.* 100 per cent) of citrate solution, kept at 60° C. for twenty-four hours and then filtered as before. A jelly-film was made thus: To a

tube of 5 cc. of "coefficient jelly," 0.5 cc. (5 units) of stain and 0.8 cc. of alkali solution (8 units) were added, together with 3 cc. of the 100-per-cent extract, and the content of the tube was made up to a total of 10 cc. by 0.7 cc. of water. The jelly was boiled and a film made from it in the usual way, fresh citrated blood being placed on it. The object was to see whether this jelly would cause the rod-shaped bodies again to appear in the lymphocytes, for we believed that it was the extract which caused their appearance. It was necessary, therefore, to raise the index of diffusion of the jelly as high as possible short of killing the cells, in order to cause maximum diffusion of the contents of the jelly into the lymphocytes. The coefficient of diffusion of these cells is 14, and we added one more unit of alkali to the jelly in order to cause the extract to diffuse to the utmost into the cells. This is the equation:

$$cf = (5s + 8a + 1.5x + 7h + t) - (6c + 1.5n) = 15.$$

where  $x$  is the 3 cc. of extract which is alkaline to the extent of about 1.5 units, and contains 3 per cent (3 units) of sodium citrate and 1 per cent (0.5 unit) of sodium chloride.

Several fields of the specimen were first looked at and the ordinary resting condition of the lymphocytes noted. The slide was then placed in the 37° C. incubator for eight minutes. The same fields (containing the same lymphocytes) were then again examined, and pictures were seen which had never been seen before, for nearly every lymphocyte in the specimen was

unquestionably in the act of reproducing itself by mitosis.

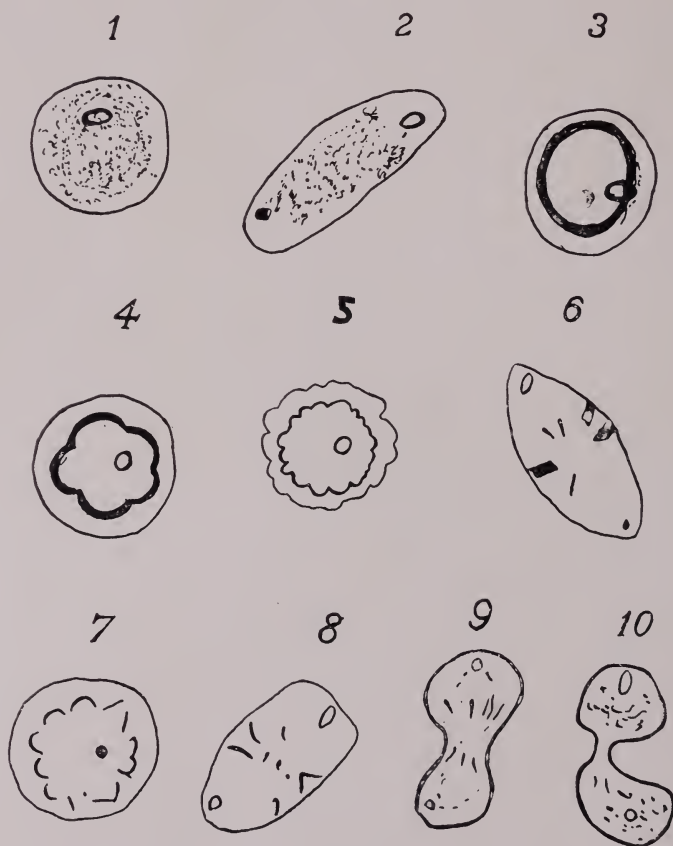
If any doubt existed as to whether the rod-shaped bodies which had been seen in the cytoplasm of the cells were really chromosomes, that doubt was now set at rest. The cells were certainly not reproducing themselves when they were first placed on the jelly-film; but after they had absorbed the contents of the jelly during the eight-minutes' incubation at  $37^{\circ}$  C., they gradually went through the process of cell-division by mitosis, and on the removal of the slide from the incubator they were found in the act of reproduction with their chromosomes and centrosomes stained bright scarlet.<sup>1</sup>

These mitotic divisions, induced for the first time in living human cells, revealed the fact that the phenomenon of mitosis in lymphocytes differed in many details from the commonly accepted ideas regarding karyokinesis which have been adopted from the study (with the older fixation methods) of dead cells other than lymphocytes. The nucleus does not vanish; it forms the spindle. The chromosomes are not derived from within the nucleus, but are formed from the normal Altmann's granules which exist in the cytoplasm. The centrosomes are not mere "dots" at the poles of the spindle, but are derived from the nucleolus which has divided into two.

Fresh films were made, and bloods taken from other

<sup>1</sup> That division had been seen in lymphocytes with this jelly, and some of the facts which led up to this discovery were published by us in *The British Medical Journal*, October 23, 1909.

persons were tried, and before long hundreds of mitotic figures were induced in lymphocytes, some of which closely resembled the karyokinesis, as described in the diagrams and drawings in well-known books on Cytology. The Altmann's granules, however, always form the chromosomes,<sup>1</sup> the nuclear wall forms the spindle, and the nucleolus forms the centrosomes. Thus:



<sup>1</sup> The chromosomes of lymphocytes do not always appear as definite "rods," but may look as if they were composed of masses of granules. See photos.

As will be shown in the succeeding chapters, one can now induce mitosis in lymphocytes whenever one pleases, and we have seen all stages of their cell-division. It must be remembered that to induce all these stages occupied many months of work, and involved the employment of many varieties of the jelly-films. I shall now describe these divisions in detail, because we have since been able to induce divisions in other human cells, and therefore there is reason to believe that the phenomenon of mitosis in other varieties of cells is similar, if not identical, with that of lymphocytes, especially as regards the Altmann's granules forming the chromosomes and the nuclear wall forming the spindle, both of which are important cytological points.

The normal lymphocyte (figs. 40-2) occurs in a great variety of sizes in the body. In the blood one usually sees the smaller sizes, but in the glands (and not only in the lymphatic glands) the cell may reach large proportions. As will be shown later, it is quite a different class of cell, cytologically, from the so-called polymorphonuclear leucocyte, and it must spend only a portion of its life in the peripheral circulation. The lymphocyte has a large round or kidney-shaped nucleus, within which there are one or two nucleoli. In the living cell the nucleus appears to be a transparent membrane (fig. 40) which stains a faint blue with Unna's polychrome dye,<sup>1</sup> and it is tucked in at its poles to be attached to the nucleolus. Outside the nucleus, and studded on its surface, a

<sup>1</sup> Chromatin stains scarlet.



large number of chromatin granules (figs. 41-2) are found which really are in the clear cytoplasm, and they are frequently extruded with the cytoplasm into the pseudopodia, especially if amœboid movements are excited by atropine. When a lymphocyte "flagellates," these granules are thrust out through the cell-wall and become separated. When the cells are on jelly which makes them divide, amœboid movements cease, and then the procedure is as follows: The nucleolus, which is shaped like a minute ring, and stains as if it was composed of chromatin, splits either into two rings<sup>1</sup> (figs. 43-4), or into two dots of chromatin which form the centrosomes. They then separate and emerge at opposite poles of the cell out through the mass of granules in the cytoplasm (figs. 45-7), and in doing this they seem to pull out the nucleus into the form of a spindle. The chromatin granules of the cytoplasm in the meantime are gradually collected into masses round the waist of the spindle (fig. 44), and ultimately they form a belt of chromatin round it on its outside (figs. 48-9). In a specimen in which one can see down through the spindle it will be observed that this belt divides into a number of chromosomes (figs. 50-1), which become semilunar-shaped with their points inwards (figs. 52-5). Each chromosome is in contact with its neighbours at its points (figs. 62-3). Each one of them then divides into two (fig. 64). One half of every chromosome travels towards one nucleolus-centrosome,

<sup>1</sup>One of us (J. W. C.) has recently seen a ring-shaped centrosome in the act of division. It appeared hour-glass shaped.

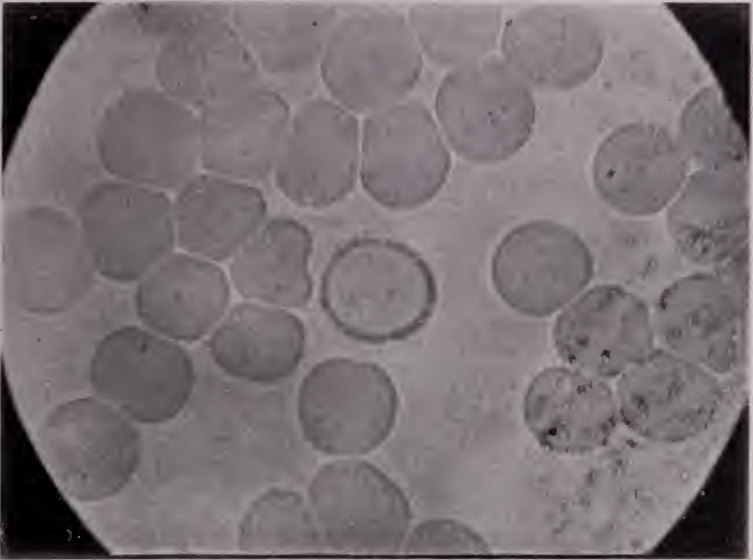


FIG. 40.—A resting lymphocyte. Note the deeply stained masses of granules in the cytoplasm, which is bulged out in places. The large transparent nucleus and the stained ring-shaped nucleolus can also be seen.

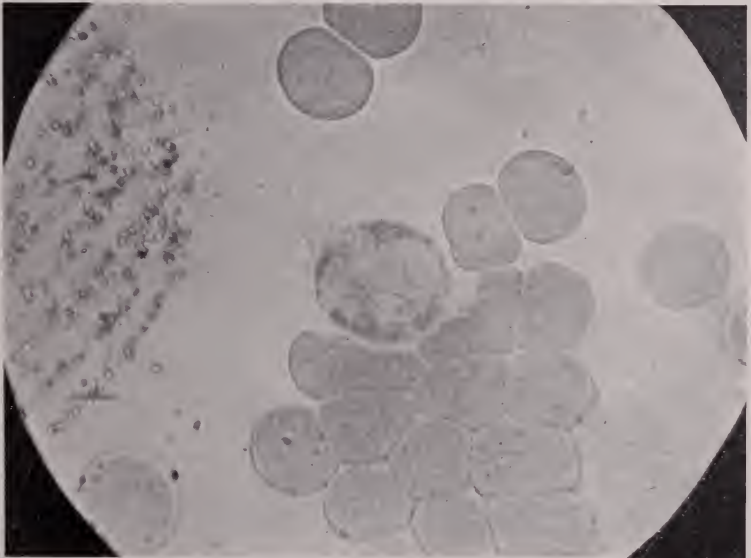


FIG. 41.—A resting lymphocyte. The Altmann's granules in the cytoplasm are stained.



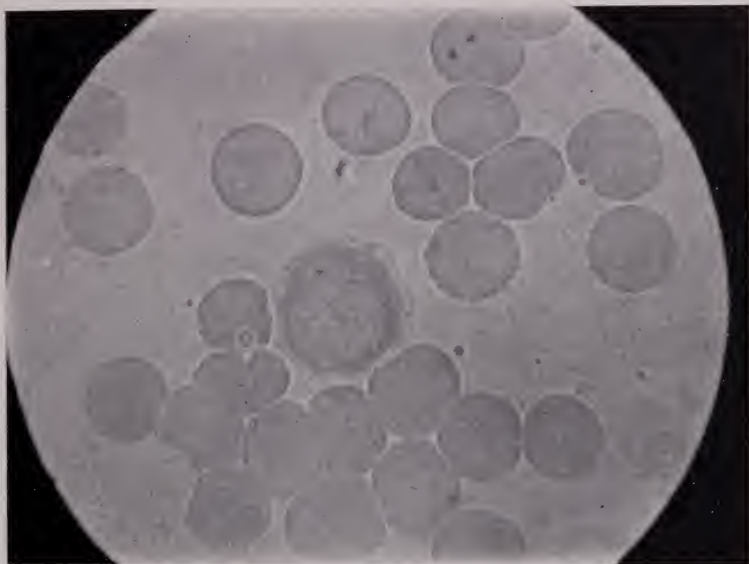


FIG. 42.—A resting lymphocyte. The cytoplasm, the granules, the nucleus, and the nucleolus can be distinguished.

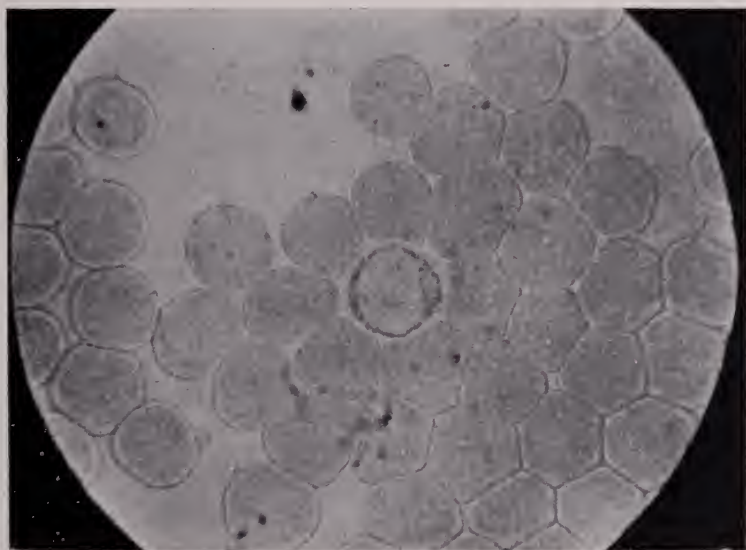


Fig. 43.—The earliest stage of mitosis. The nucleolus has divided into two rings.



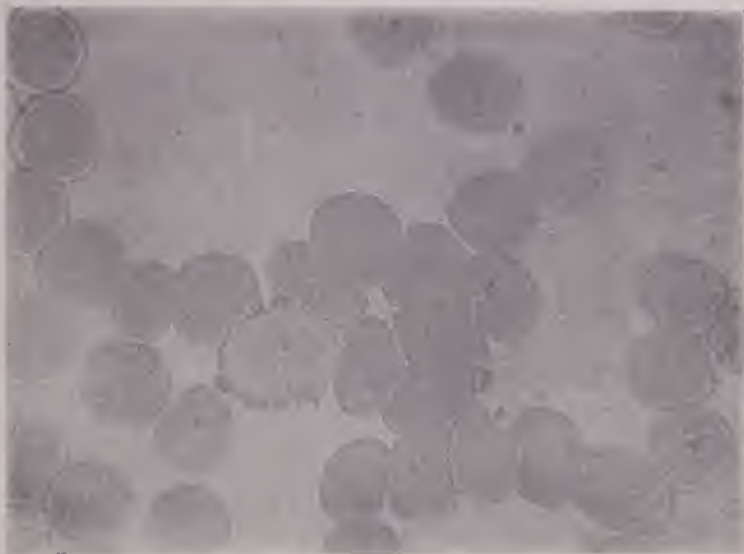


FIG. 44.—Early mitosis in a lymphocyte. Looking down through the spindle (polar aspect). The nucleolus has divided into two centrosomes, each of which is ring-shaped. The spindle is surrounded by a belt of chromatin granules.

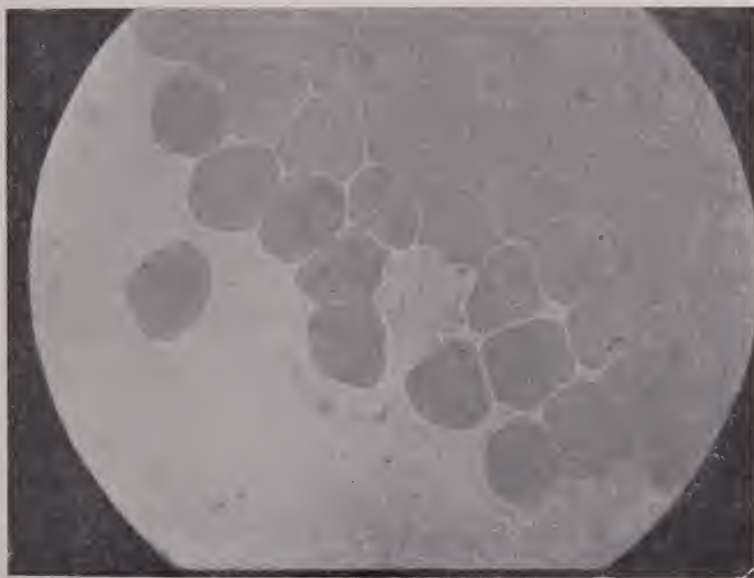


FIG. 45.—Mitosis in a lymphocyte. Profile aspect. The two ring-shaped centrosomes can just be seen towards the poles. The granules are becoming formed into chromosomes.





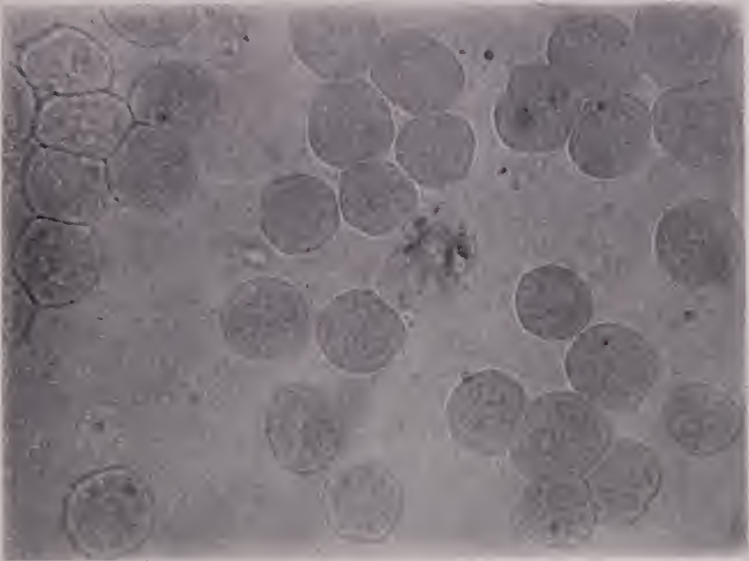


FIG. 46.—Foreshortened appearance of a mitotic figure in a lymphocyte. The position of one nucleolus-centrosome at the pole of the figure is well shown.



FIG. 47.—Profile aspect of mitosis in a lymphocyte. The relative positions of the centrosomes and chromosomes can be seen.





FIG. 48.—Profile aspect of mitosis. The belt of chromatin is formed round the waist of the cell.

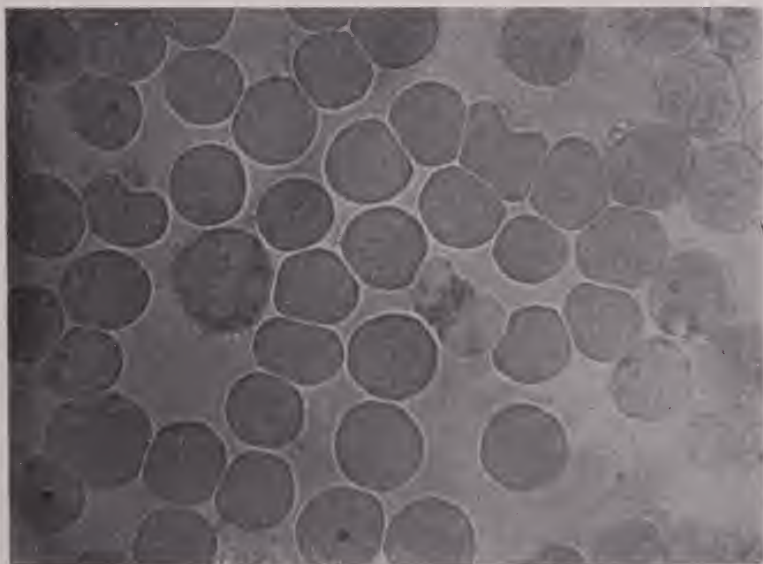


FIG. 49.—One resting and one dividing lymphocyte. In the latter the chromosomes are beginning to divide. The centrosomes appear as dots of chromatin.



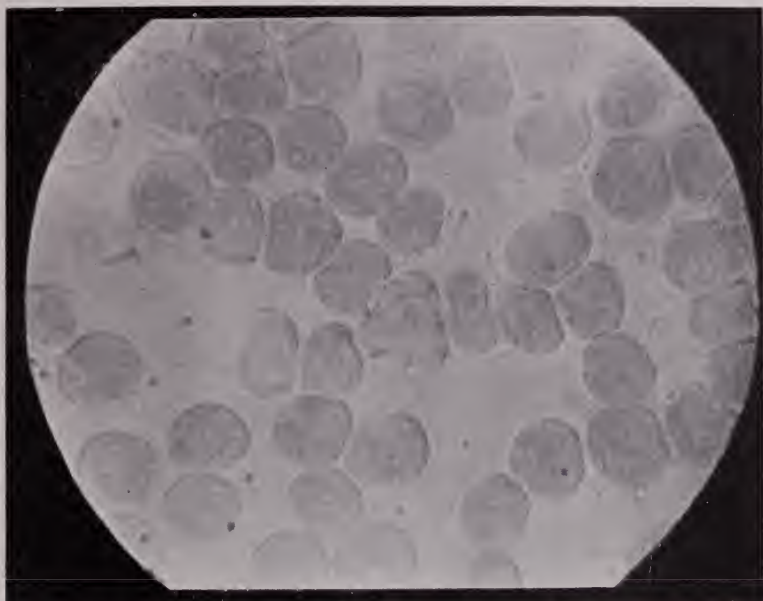


FIG. 50.—Polar aspect. The belt of chromatin granules is dividing into chromosomes.

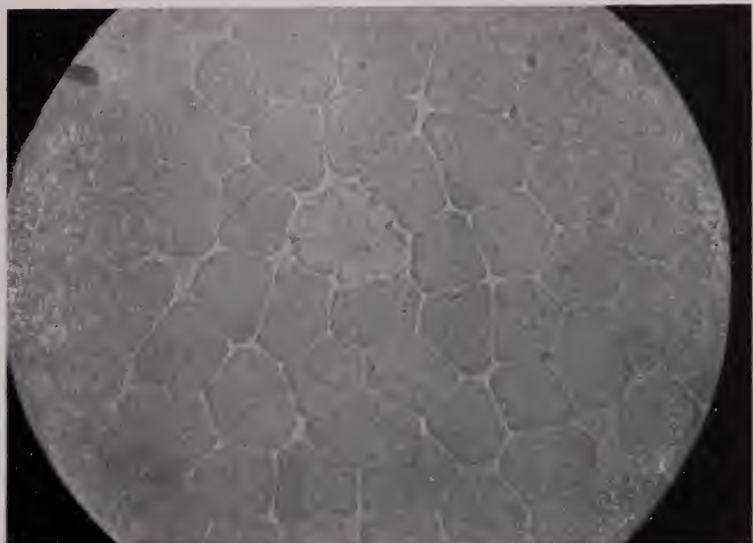


FIG. 51.—Polar aspect. The chromosomes are becoming semicircular.





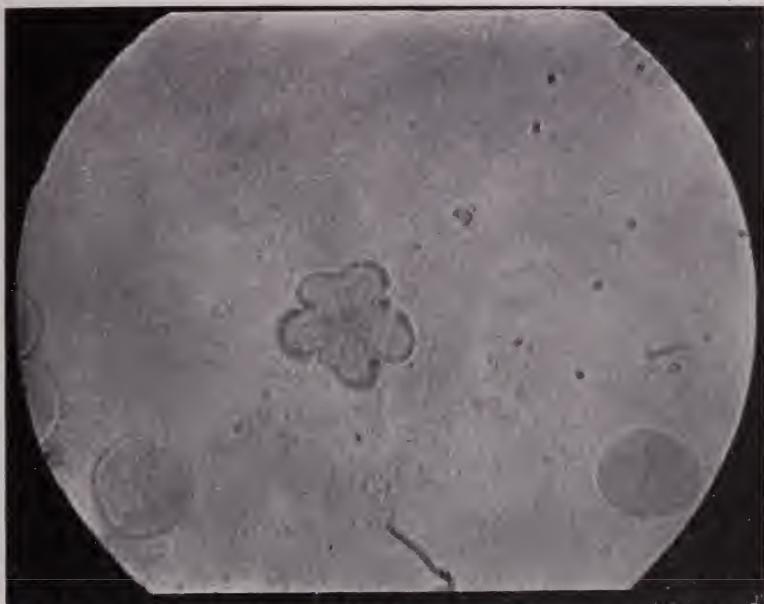


FIG. 52.—Polar aspect. An "aster" stage of mitosis in a lymphocyte.

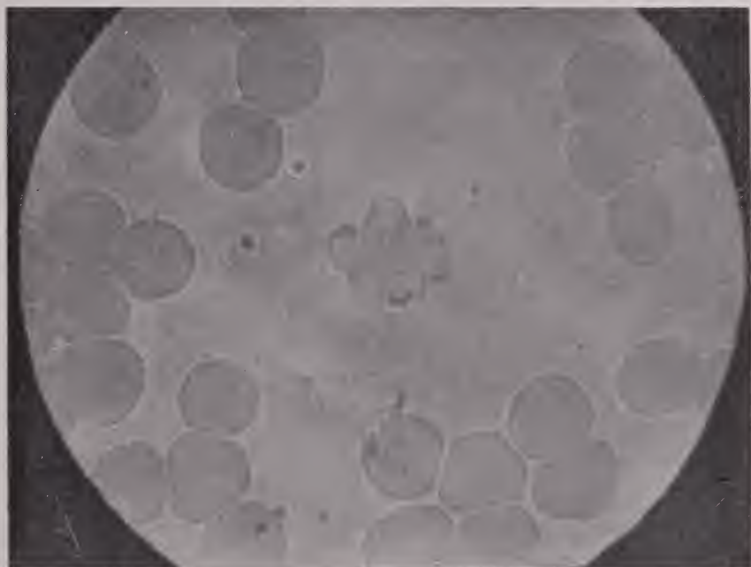


FIG. 53.—Polar aspect. Some of the chromosomes are semicircular-shaped; some are dots of chromatin.





FIG. 54.—Polar aspect. One centrosome can be seen at the pole of the "aster" figure.

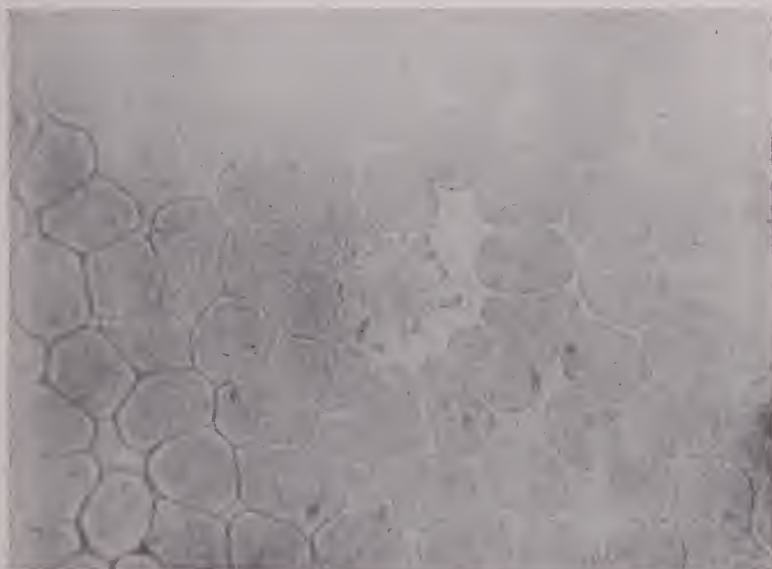


FIG. 55.—Polar aspect. Sixteen chromosomes could be counted in this cell.





FIG. 56.—Profile aspect of mitosis in a lymphocyte.

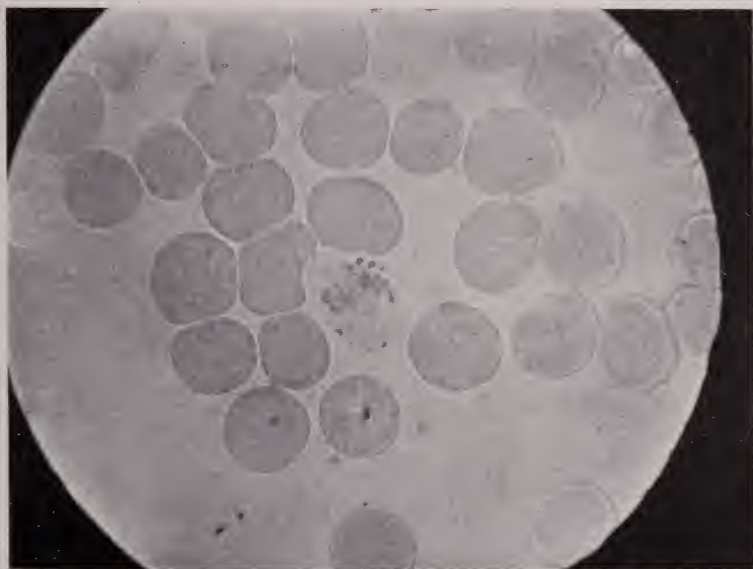


FIG. 57.—Profile aspect of mitosis in a lymphocyte.





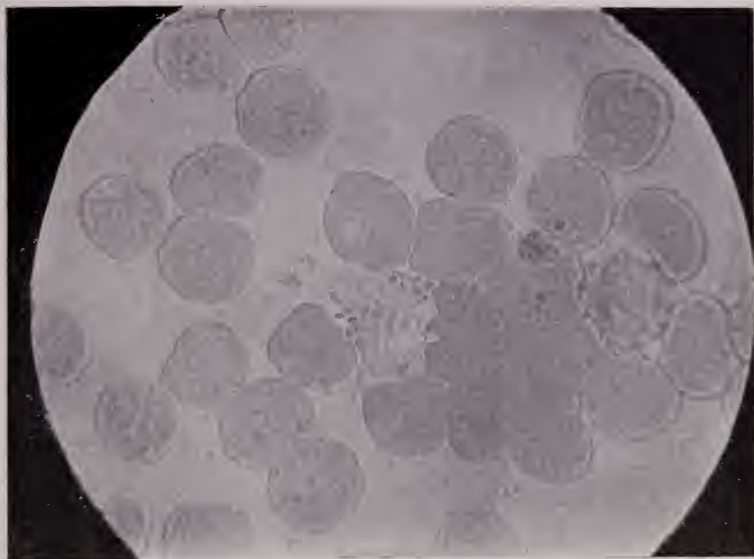


FIG. 58.—Profile aspect. The chromosomes can be seen at the waist of the spindle.

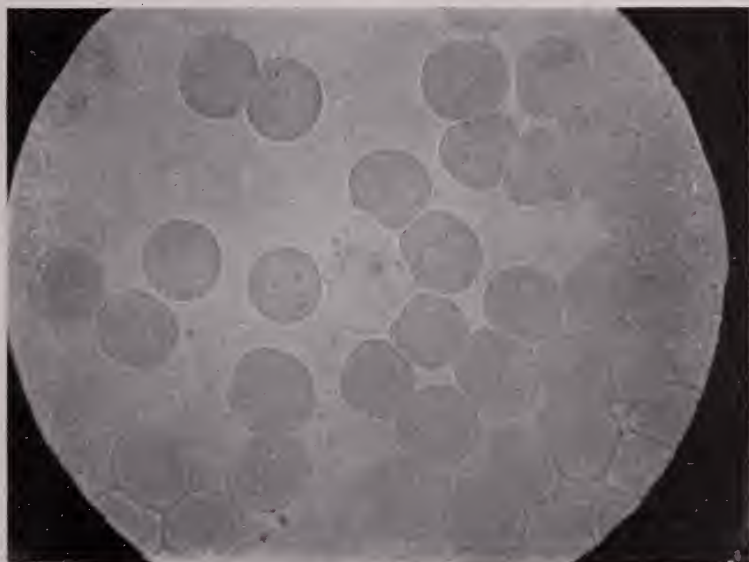


FIG. 59.—Profile aspect. A figure frequently seen.



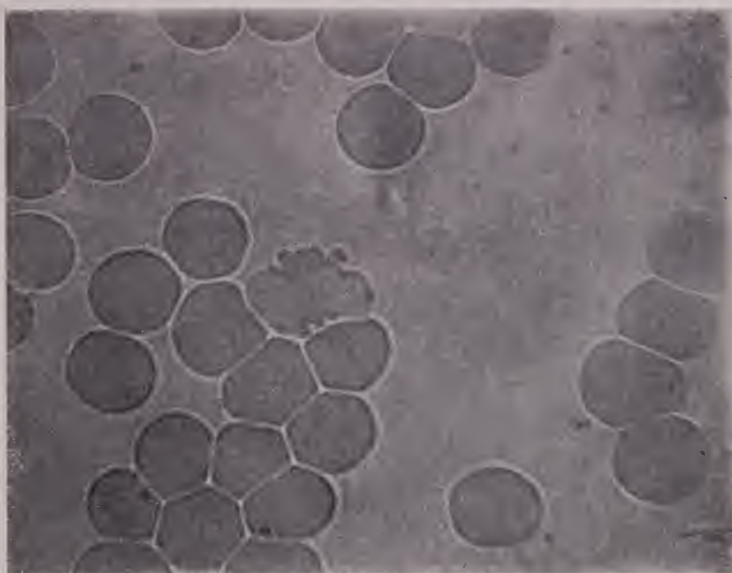


FIG. 60.—Profile aspect of mitosis.



FIG. 61.—Oblique aspect of mitosis in a lymphocyte.





FIG. 62.—Polar aspect of mitosis in a large lymphocyte from a patient suffering from carcinoma. There are sixteen chromosomes.

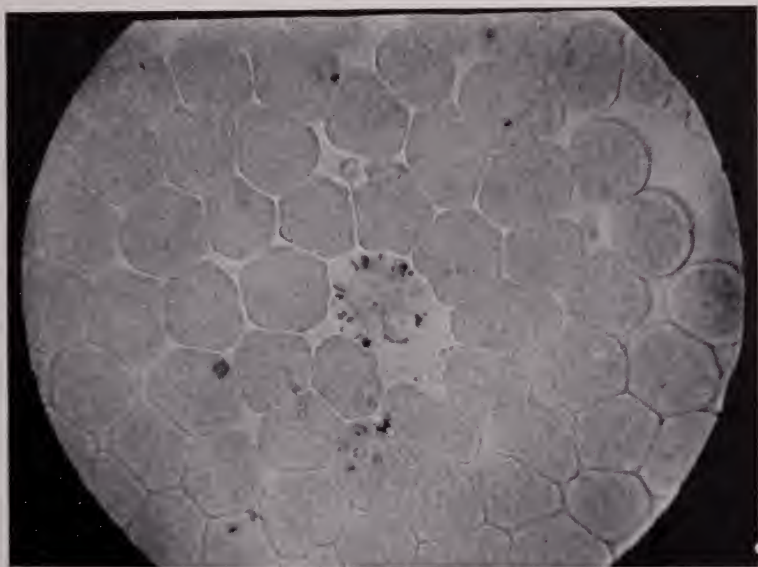


FIG. 63.—Polar aspect. The chromosomes were V-shaped with their apices inwards to be attached to the nucleus-spindle, which can dimly be made out.







FIG. 64.—Polar aspect of mitosis in a large lymphocyte from a cancer patient. The chromosomes are dividing.

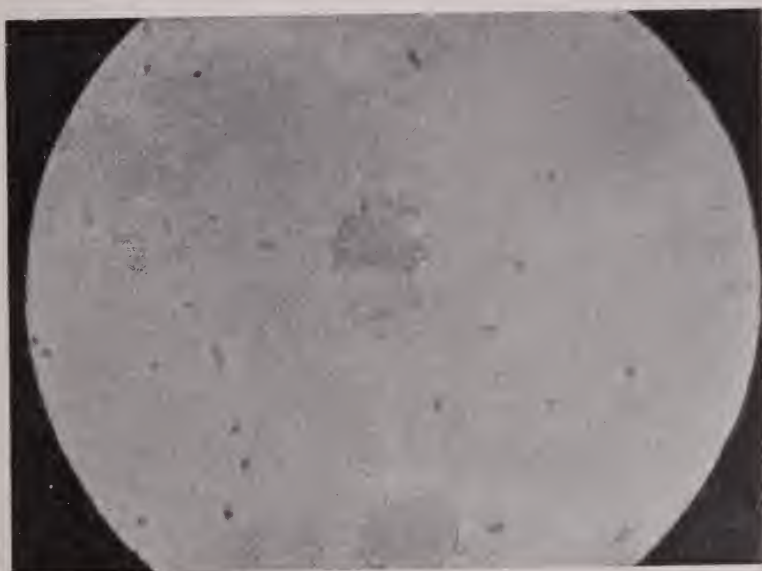


FIG. 65.—Profile aspect of mitosis.



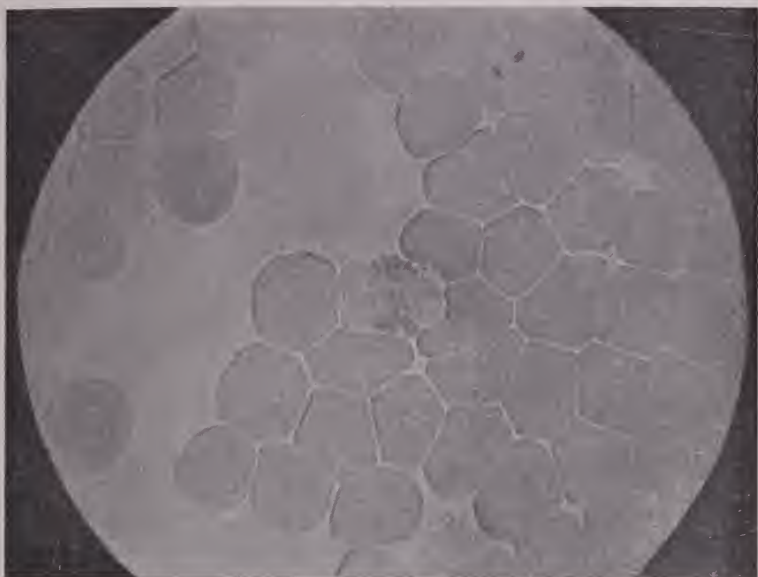


FIG. 66.—Profile aspect. The figure is fully formed. One nucleolus-centrosome is ring-shaped; the other is a dot of chromatin.

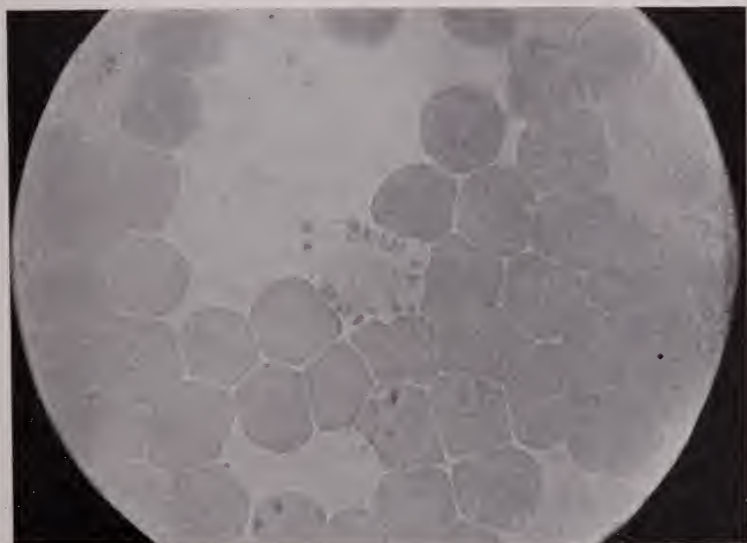


FIG. 67.—Profile aspect. The sixteen chromosomes could be counted.





FIG. 68.—The cell has become constricted in its centre.

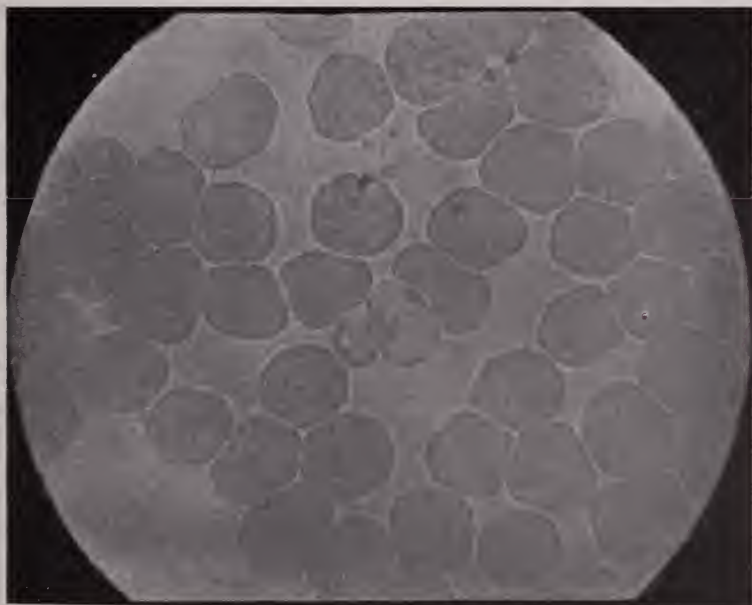


FIG. 69.—Profile aspect. Complete division is about to occur. The chromosomes are being reconverted into granules, but the mitotic figure is not quite finished at the dividing-point.





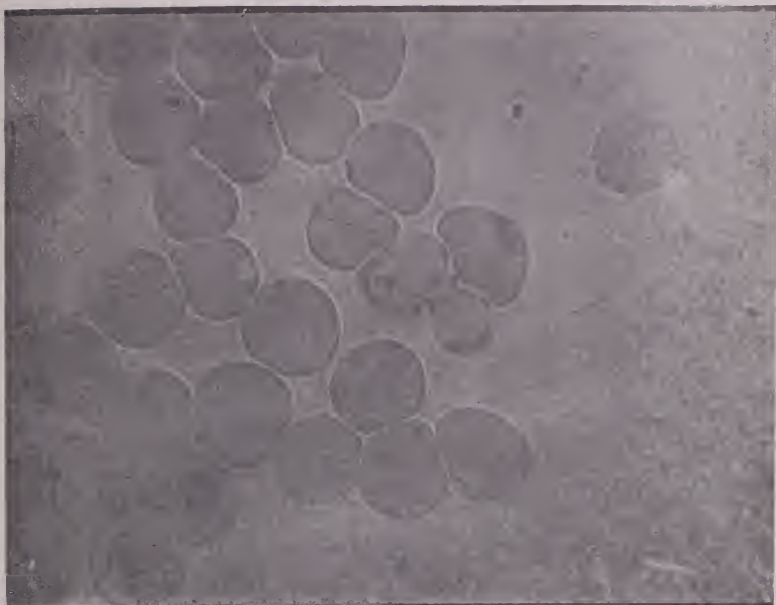


FIG. 70.—Profile aspect. The spindle and chromosomes have divided, but the cell wall has not yet separated.



FIG. 71.—Completion of mitosis in a lymphocyte.



and the other half towards the other centrosome (figs. 56-61, 65-7). The spindle divides in the centre (figs. 68-70); and lastly the cell itself divides (fig. 71). In each daughter cell the chromosomes return to their granular condition and pervade the whole cytoplasm. The single centrosome (for there is now one only in each daughter cell) again becomes tucked into the centre of the transparent nucleus—which consists of one half of the original spindle, and thus the cycle of mitosis is completed. Doubtless each chromosome granule divides during some part of the cycle, but owing to their minute size we have not been able to see their division.

The cells, of course, do not usually divide in definite stages such as the aster and diaster, although sometimes a cell will be found which presents one of them. Sometimes one sees that the chromosomes may be dividing in one part of the cell, while some chromosomes in another part are being reconverted into granules of chromatin. The way in which a cell is lying on the jelly must be taken into consideration in the determination of the stage of mitosis. One rarely finds a perfect figure as described in diagrammatic drawings of other types of cell, for the cells frequently appear foreshortened owing to the oblique manner in which they happen to come to rest under the cover-glass. The position of the ring-shaped nucleolus-centrosomes is of prime importance in the determination of the stage of the mitotic figure.

In observing any stage of mitosis, however, it will

be seen at once—a point on which we must lay stress—that the chromosomes are *outside* the nucleus are formed by the conglomeration of the Altmann's granules in the cytoplasm. As will be shown later on, this is also the rule in leucocytes and some epithelial cells as well as in lymphocytes.

The phenomenon of mitosis, then, as seen in these cells when they are stained alive, differs very materially from the usual descriptions of it as seen in cells which have been killed, fixed, cut into sections or otherwise manipulated, and stained. The old idea was—although divisions had not been seen actually in lymphocytes—that the chromosomes were formed out of some chromatin which is within the nucleus, and that, inside this again, a spindle, which does not exist in the resting stage, is formed. The nuclear wall was described as vanishing during mitosis according to most conceptions. But, as I have described, mitosis is a much simpler phenomenon.

The misconception has been due, I think, to several factors. In the first place most cytological research has been carried out with plant-cells, and animal cytology has arisen from it. In the second place, cells up to now have only been caught in the act of mitosis; their cycle of cell-division has not been followed from the resting stage to completed division in one individual cell. The morphological elements of a resting cell have been studied, and then those in one killed in the act of division, and the part played by each element has been deduced from its new position—not watched throughout. Lastly, owing to manipulation, the so-called

Altmann's granules of some cells have been crushed into the nucleus, in which case they look as if they formed part of it, or were inside it—a fallacy which has given rise to great controversy regarding the nature of these granules, to the statement that they do not exist in some cells, *e.g.* lymphocytes and cancer cells, and to failure of appreciation of the fact that the chromosomes are formed out of them. Let mitosis be induced in a living cell and no second glance will be required to realise the real sequence of events.

The mitosis of plant-cells seems to go on within the nuclear wall, but this is not the case in the animal cells which we have seen. The granules in the cytoplasm of Altmann's granules are larger in some classes of cells than in others. For instance, they are much larger in eosinophile leucocytes than in lymphocytes. When they are large their position is obvious, but when they are small, as in lymphocytes and cancer-cells, during the killing of the cell as it is fixed the small granules—which are composed of chromatin—adhere to and are merged into the nucleus. No matter how we try to fix a specimen, death takes time, and the liquefying cytoplasm bulges out the cell-wall. The rapidity of death depends upon the diffusion of the fixative into the cell, and this diffusion takes time. Hence when a cell is stained and fixed, it appears as if its nucleus is a mass of chromatin—which it is not—and its halo of cytoplasm, which has bulged out of the cell-wall, is now apparently devoid of Altmann's granules. This is a pitfall into which we fell ourselves, for, although we had seen the granules of



lymphocytes and cancer-cells forming the chromosomes, we thought that these granules were composed of chromatin, but that Altmann's granules as exemplified in polynuclear leucocytes were of quite a different nature. As will be shown later, the granules of leucocytes also form the chromosomes in the same way as those of lymphocytes and cancer-cells. Professor Lorraine Smith suggested that the apparent absence of granules in lymphocytes and cancer-cells might be due to the fixative, and he is right. The cytoplasm of every living lymphocyte is full of minute granules which stain like chromatin with aniline dyes, and these granules clump together to form the chromosomes during cell-division—a point about which there can be no question whatever.

In some other cells, such as some large cells of the liver, we have seen large granules in the cytoplasm (as well as fat globules), which will not stain. What their function is we do not know, for we have not been able to induce divisions in these cells. The granules of lymphocytes we shall henceforth style "chromosome-granules," the nucleolus as the "nucleolus-centrosome," and the nucleus as the "nucleus-spindle."

## CHAPTER XI

THE DIVISION OF LYMPHOCYTES INDUCED BY THE ANILINE DYE—THE AUGMENTING ACTION OF ATROPINE AND EXTRACT OF HÆMAL GLAND—"AUXETICS"—THE CYCLE OF CELL-DIVISION—THE POSSIBILITIES OF THE INDUCED CELL-DIVISION BEING DUE TO "DEATH-STRUGGLES"—ASYMMETRICAL AND REDUCED DIVISIONS

THE fact that mitotic figures could be made to appear in lymphocytes was very satisfactory, for it seemed to us to be a step in the solution of the problem of the cause of the multiplication of cells. It was true that we had only seen them in lymphocytes; but still these mitotic divisions had occurred in response to the action of a chemical substance, and if these cells were capable of dividing in response to it, it appeared reasonable to suppose that other cells would do the same and that it was possible that they would *only* divide when they absorbed a chemical substance. We believed at first, of course, that the substance which had induced the divisions was the extract of the dead hæmal gland; but before many experiments had been made this suggestion received a check. One day a cell was seen

stained in an early stage of mitosis; its ring-shaped nucleolus-centrosome was lying at a pole of the nucleus-spindle in the cytoplasm, outside the mass of granules which had not yet collected round the waist of the cell. Now when this early figure was seen by me, I remembered that I had seen something very similar to it before, and on turning up a paper (*British Medical Journal*, January 16, 1909), which described some work done more than a year previously, it was found there mentioned that the nucleoli sometimes appeared outside the nucleus in the cytoplasm. Now, this position of the nucleolus-centrosome is the first step in mitosis, and therefore it was grasped that this mitosis must have been seen before, although the fact was not realised at the time. Another far more important point was also grasped, viz. that when the mitosis had been seen a year previously, no extract of hæmal or any gland had been either used or thought of.

The notes of the previous work were referred to, and it was found that when the—as it turned out—early stage of mitosis had been seen, the cells had been resting on a jelly which contained only Unna's stain and atropine. It was clear, therefore, that either one or both these substances would induce divisions in lymphocytes and our hopes were rather damped, for both these substances, unlike the extract of hæmal gland, are entirely artificial, and could not possibly be concerned in the cell-proliferation of healing.

Each of the ingredients of the jelly—described in the last chapter—which induced well-marked mitosis in lymphocytes was now tried separately. Jellies were

prepared which contained each of them in turn, and jellies were prepared which contained only the salts sodium citrate and sodium chloride. Many experiments were made from each, and several different strengths of the different substances were tried repeatedly on fresh lymphocytes. It was thus ascertained that Unna's polychrome methylene blue (Grubler) contains some substance which will induce divisions in lymphocytes. It requires a high concentration of this stain for this purpose, and this was the reason why advanced divisions had not been seen in the several years' previous work with this dye. Unless the jelly contained Unna's stain, no mitosis whatever would occur. Repeatedly they were tried, but none of the other ingredients by themselves could be made at that time to cause lymphocytes to reproduce themselves. The 100-per-cent extract of hæmal gland by itself certainly did not do so, nor did the atropine; but both the extract and atropine—and this was an important point—greatly augmented the action of the stain in inducing mitosis. By itself at least 10 units (1 cc.) of polychrome stain were required to induce mitosis; but if a certain quantity of atropine or of extract, or, better still, of both, was also added to the jelly, one could cause advanced mitosis in lymphocytes with only two or three units of stain. It was a remarkable state of affairs that neither atropine nor extract would induce divisions by themselves, but that they augmented the action of the stain in doing so to a very marked degree.

During all this experimentation, which occupied a

considerable time, many points connected with the process of inducing divisions were learnt. We had three factors to deal with, viz. polychrome dye, atropine, and extract consisting of the soluble remains of dead hæmal gland of 100 per cent. It was found that lymphocytes would not make any attempt whatever to divide unless they absorbed some of the polychrome stain. As the stain passed into the cells, it stained first their chromosome-granules and their nucleolus-centrosomes. Like polynuclear leucocytes, lymphocytes do not appear to suffer much harm to their lives while their granules are stained, but as soon as their nucleolus-centrosomes are reached by the dye death occurs. Mitosis takes place about the time when the granules are staining, and therefore the rapidity of the onset of mitosis depends on the rapidity of the diffusion of the dye into the cells. It is thus evident that the gradual diffusion of the stain first causes mitosis and then death because it kills the cells by combining with and staining the nucleolus-centrosome. The rapidity of the diffusion of the stain is increased by concentrating it, by the presence of alkalies, or by heat. These factors also hasten death and they likewise hasten cell-division. With regard to the factor heat, however, we must add the qualification that no lymphocyte will divide below a temperature of  $30^{\circ}$  C. or above about  $40^{\circ}$  C., and for this reason we have employed a temperature of  $37^{\circ}$  C. throughout these experiments for inducing division.

Now, mitosis is a process which occupies a certain amount of time. If the diffusion of the stain is very slow, the time taken by the act of mitosis is

correspondingly slow. But as far as we can see, mitosis cannot occur completely in less than about three minutes. It can take a very long time in its accomplishment; but it cannot be completed in *less* than three minutes. Hence, if mitosis can take place slowly, without the cell being killed by the stain, complete mitosis can occur; but if the nucleolus-centrosome stains in less than a minute or so, death will occur before the cell has had time to divide. This fact governs the whole of this experimentation, for when inducing cell-division with the aniline dye it must be remembered that the mitosis has to occur after the cell-granules have begun to stain, but before death is occasioned by the staining of the nucleolus-centrosome.

We have the power of accelerating and delaying the diffusion of the stain into the cells by adding or subtracting alkali, or by increasing or decreasing the concentration of the stain by rules which can be plotted in an equation, and therefore by such an equation we can ascertain the rate of cell-division as induced by the chemical agent. But throughout it must be appreciated that it is the stain which is inducing the cell-division, and that if the stain is not sufficiently concentrated no division will occur at all. On the other hand, it must also be remembered that an excess of stain will poison the cells too quickly. A cell must absorb a certain amount of stain before it will divide, and the absorption depends on the concentration of the stain in the jelly and on the alkali. One may place living blood-cells on a jelly which contains the best ingredients for inducing cell-division; but unless the



alkali is correct according to the equation—that is to say, unless the index of diffusion of the jelly is correct for the coefficient of diffusion of the cells, the latter will take no notice whatever of the mitosis inducing agents in their surroundings. But make diffusion factors of the jelly right and the cells will then respond immediately, and as many as 90 per cent of the lymphocytes in a specimen may be made to divide.

Not only does the rapidity of the onset of mitosis depend on the physical laws of the diffusion of substances into cells, but the actual stage reached in a given cycle of cell-division also depends on them; for the completion of the mitotic cycle occupies a certain amount of time, which varies inversely with the quantity of the stain absorbed by the cell, and this absorption depends on the coefficient of diffusion, heat, alkali, etc. The following experiment illustrates this point. A jelly-film was made which induced almost completed divisions in lymphocytes in ten minutes. By making several films and removing them, one at a time, from the 37° C. incubator at each minute, it was seen that mitosis began with the staining of the granules at about the seventh minute, and that death occurred at about the ninth. The experiment was repeated, and at the seventh minute, immediately while mitosis was occurring, the slide was quickly removed from the 37° C. incubator to one which maintained 32° C. The sudden lowering of the temperature delayed the diffusion of the stain into the cells, and the interesting point is that the mitosis ceased when the diffusion of the stain was suddenly arrested, and the cells died

slowly. Twenty minutes afterwards, when all the chromatin was stained, it was seen that the mitosis had been arrested in those early stages reached at the seventh minute.

Thus it appears from this experimentation that not only will a lymphocyte not reproduce itself *in vitro* unless it absorbs a chemical "exciter of reproduction," but also the actual stage reached in its act of mitosis varies directly with the quantity of that substance which has diffused into the cell. It follows that, *in vitro*, before a cell will reproduce itself *completely* it must receive a definite quantity or dose of the chemical substance.

In addition to the above factors, the divisions of the cells depend upon their vitality. If some blood is citrated and kept for two days, it is very difficult to induce divisions in the lymphocytes. The longer cells have been shed the slower they are to respond to the division-inducing action of the stain, in spite of the fact that their coefficient of diffusion has fallen. It is impossible to induce divisions in cells with auxetic jelly if other cells from the same sample of blood will not show excited movements on kinetic jelly.

The foregoing points showed that the reproduction of lymphocytes *in vitro* depended entirely on the aniline dye. The dye did not merely increase the cells' propensity to divide; it actually caused the division. Lymphocytes had never been seen to divide before, and they certainly will not divide *in vitro* unless one takes deliberate steps to make them do so. Mitosis is a complex phenomenon which only occurs as an act of

cell-reproduction, and *in vitro* the only way to cause it to take place was to force the cells to absorb the chemical "exciter of reproduction" contained in the aniline dye. It appeared reasonable to us to suppose that there might be other "exciters of reproduction," not only for lymphocytes, but for other cells as well, and therefore we proposed to call the substance in the aniline dye which caused cell-division in lymphocytes an "auxetic" (*αυξητικός*, an exciter of reproduction), a convenient term suggested by Professor Harvey Gibson, which might be applied to other substances having a similar action if such were proved to exist.

The next steps were to investigate the "augmenting" actions of both atropine and the extract of hæmal gland. It has been pointed out how atropine, being an alkaloid, greatly excites amœboid movements in lymphocytes and leucocytes, and it was soon seen that atropine also greatly augments the action of the polychrome dye in inducing mitotic figures in lymphocytes. The best strength of atropine to be added to the jelly which contains the stain is that which causes maximum excitation of amœboid movements. If this is done lymphocytes can be caused to divide with the strength of the stain reduced to one-fifth of the minimum amount of it which will, by itself, induce mitotic figures. In other words, atropine will not by itself induce divisions on the microscope slide, but it will augment the "reproducing" action of polychrome stain five-fold. Another point was also noticed, which was very material to the main object of these researches, in that stain, *plus atropine*, caused lymphocytes to



FIG. 72.—Asymmetrical mitosis in a lymphocyte induced by azur stain augmented by atropine.

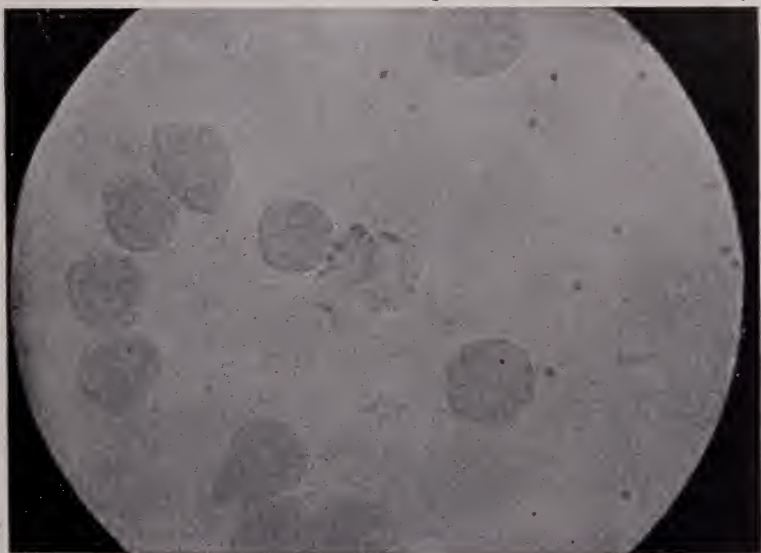


FIG. 73.—Asymmetrical mitosis induced by azur stain augmented by atropine.



undergo curious one-sided mitoses in some instances (figs. 72, 73).

We now investigated the "augmenting" action of the extract of hæmal gland. This was even more powerful than that of atropine. So great was it that one can employ a jelly which only contains three units of polychrome stain—which will never induce divisions by itself; and if 3 cc. of the 100-per-cent extract of dead hæmal gland is also contained in the jelly, complete divisions can be induced in lymphocytes without the cells actually being coloured by the stain at all. Yet all attempts at this stage to cause the extract to induce divisions by itself had failed.

Thus, by means of a mixture of a little stain, say 4 units, 0.7 cc. of a 1-per-cent solution of atropine sulphate, 3 cc. of the 100-per-cent extract of hæmal gland, 6 units of alkali solution, and 0.3 cc. of water added to 5 cc. of coefficient jelly to make a total of 10 cc., one can induce advanced divisions in lymphocytes, without the cells staining at all in ten minutes.

$$cf = (4s + 6a + 1.5x + 7h + t) - (6c + 1.5n) + 0.5z = 11.5.$$

We have already stated that mitosis occurs about the time when the stain has diffused into the cells sufficiently to stain the granules. But now with the combination of stain and the augmenting substances mitosis will occur without the stain colouring the granules at all. In spite of this, however, stain is essential. Hence we suggest the theory that the stain induces divisions by acting on the chromosome



granules; but that, since it is not necessary for it actually to colour these granules, as shown by the last experiment, it seems probable that the stain induces divisions by virtue of some substance contained in it which does not colour granules. It is not the stain itself which induces divisions; it is some constituent of it, and the action of that constituent is greatly augmented by atropine and extract.

The next point is that when mitosis is induced on a microscope slide with stain, death is premature. Even if there is not sufficient stain to colour the nucleolus-centrosomes, death rapidly follows. We believe that this dye contains at least two constituents which can be utilised differently by the cell's protoplasm—a substance which, by combining with the cell-granules, causes the cell to reproduce itself, and a poison which kills it. Both diffuse into the cell together; mitosis is induced and then the cell dies prematurely. If the stain is sufficiently concentrated, the chromatin after it is dead will combine with it, and the chromatin then turns bright scarlet. From prolonged observation of these induced divisions we think that the scarlet coloration of the chromatin is a post-mortem effect. The stain as it diffuses into the cell induces division as it combines with the granules, which die and become coloured one by one. All the time the stain is passing farther into the cell, and later and later stages of mitosis are being induced. Ultimately the nucleolus-centrosome is reached and the cell dies; and thus it is seen dead in the act of mitosis with its chromosomes and centrosomes stained bright scarlet. If, on the other

hand, the concentration of the stain is reduced and its action augmented by atropine and extract, still the poison, but in less strength, passes into the cell; and although mitosis occurs to an advanced degree, nevertheless premature death occurs in spite of the fact that there is not sufficient strength of colouring matter to give rise to the post-mortem coloration of the chromosomes and centrosomes. Death is a gradual process—presumably it is molecular as well as cellular, for the post-mortem scarlet coloration occurs gradually; but it is not until the nucleolus-centrosome is reached that all mitosis ceases. One cannot excite amœboid movements in a cell which has its nucleolus stained.

Since Unna's polychrome methylene blue contained the active principle which caused the cells to divide, and the other two substances appeared merely to be augmenters, we now turned our attention more especially to the dye. Polychrome methylene blue stains chromatin scarlet and the nucleus-spindle a faint blue. It is made by "polychroming" methylene blue. Fresh methylene blue stains chromatin blue, and it is not so effective as the polychrome dye in inducing mitosis. The "polychroming" process consists of rendering a solution of methylene blue alkaline with sodium carbonate and naturing it for some time at a high temperature. The methylene blue turns a purple colour. This is due to decomposition—an oxidation occurs with the production of a dye known as "azur."<sup>1</sup> This azur dye can be obtained from dealers, and it can be extracted from the polychrome dye by means of

<sup>1</sup> *Centralblatt für Bakteriologie*, bd. xxix., 1901, p. 765.

chloroform. It was found that the constituent which induces divisions in lymphocytes is almost entirely confined to the azur dye. The more the azur was extracted the less efficient the polychrome dye became, and the azur is very potent although it does not stain the chromatin as well as the polychrome dye.

A concentrated solution of azur dye was made thus: In a burette 20 cc. of Unna's polychrome methylene blue (Grubler) had added to it 20 cc. of chloroform, and the mixture was allowed to stand for 12 hours. The chloroform, which sinks to the bottom, carrying some of the azur dye with it, was then run off into a shallow dish, where it was allowed to evaporate. 20 cc. more of chloroform was then added to the original 20 cc. of stain in the burette, and, after 12 hours, it, in its turn, was run off into the same dish and also allowed to evaporate. This procedure was repeated five times, and the dry azur dye was so obtained. Lastly, a solution of this dye was made by adding 5 cc. of water to the dish. This potent dye is a fluorescent red one, which, when dry, shines with a metallic lustre. A very powerful jelly for causing mitotic divisions in lymphocytes was made by substituting 0.4 cc. of this potent solution for the 0.2 cc. of Unna's stain in the last equation. By means of this jelly all stages of divisions can be readily obtained, it being only necessary to vary slightly the content of alkali in producing early or late mitosis in the ten minutes. It is better to keep at least two units of polychrome stain in the jelly, in order to stain the chromosomes more deeply.

In experimenting with this last jelly containing azur dye, an important point was found out. By delaying death as long as possible, by employing the minimum amount of alkali which will make the cells undergo mitosis, we at last succeeded in keeping the lymphocytes alive for twenty minutes, and yet mitosis was being induced during the greater part of the time. That is to say, mitosis was induced as early as possible, for, as will be shown in the next chapter, we cannot, under the experimental conditions, keep up the cell's vitality longer than twenty minutes, and it is difficult to keep them alive to divide for a longer time than even ten minutes; still their lives have been prolonged for twenty minutes.

The point revealed by this experiment was that the so-called *reduction* division is not a *special* form of mitosis in lymphocytes. The somatic number of chromosomes in the body is thirty-two, but hitherto in all the dividing lymphocytes in which it was possible to count the actual number of chromosomes their number was either sixteen or thereabouts (figs. 62, 63, 67). In other words, the divisions which we induced with the stain in ten minutes were of the reduced variety, or what Farmer, Moore, and Walker called "maiotic" divisions. By prolonging life, however, for twenty minutes, and inducing the divisions slowly, especially if only the early stages of mitosis were induced in the time, it was found that now lymphocytes divided by somatic divisions with more than sixteen and sometimes with a full number of thirty-two chromosomes (figs. 74, 75), and the statement

that the wandering cells of the body only divide by reduced divisions of the "reproductive" type is thereby disproved. In lymphocytes examined on a microscopic slide the question of the number of chromosomes seems to be entirely one of degree—it depends on the rapidity of the division, which, in its turn, depends on the quantity of the "auxetic" absorbed by the cell. By increasing the alkali one can induce divisions very quickly, provided of course there is not too much alkali. We have seen lymphocytes divide with less than sixteen chromosomes, and on one occasion, when mitosis was very rapidly induced, the number was reduced to eight only; but the number of chromosomes seems usually to remain in these round numbers, namely, thirty-two, sixteen, eight, the last one being very rare. If a division is induced in the usual way with a jelly which will kill the cells in about ten minutes, the number of chromosomes is nearly always sixteen, but a slow division will be a somatic division. There does seem, however, to be a difference in the way the chromosomes split. We have seen them in the act of splitting longitudinally, and also, and more commonly, they split transversely; although whether the longitudinal splitting is significant of a "first (heterotype) meiotic" division or not we are not in a position to state.

The asymmetrical mitoses induced when atropine is present, especially if it is present to excess, are interesting. The mitosis seems to be going on in one side of the cell. We have not seen a completed division in one of those asymmetrical mitoses, but we





FIG. 74.—An early stage of delayed mitosis induced by a jelly with a low index of diffusion. The number of chromosomes is more than sixteen.



FIG. 75.—Thirty-two chromosomes could be counted in this cell. Early mitosis delayed.





think that, from the appearance of the cells, they are about to divide into more than two daughter cells by some quite atypical arrangement of the chromosomes. The point is a very important one, for asymmetrical divisions are reported to be frequently seen in cancerous growths.

We may now summarise the facts learnt from the mitotic divisions induced in lymphocytes by the aniline dye. (1) Lymphocytes will not divide *in vitro* unless they absorb the chemical agent. (2) The rapidity of the onset of division depends on the rapidity of the diffusion of the agent. (3) The time occupied by the act of division depends on the amount of the agent absorbed and the time occupied in the diffusion of the substance into the cell. (4) If the diffusion is slow the cells divide with the somatic number of thirty-two chromosomes; but if it is rapid the number is reduced. (5) A "reduction division" means that a cell is very prolific, owing to its absorption of a large quantity of the chemical agent. (6) The rapidity of the absorption of the agent depends on the coefficient of diffusion of the cell, the concentration of the agent in the surrounding fluids, and on the presence and strength of the factors which increase or decrease the diffusion of the substances. (7) Lastly, it depends on the vitality of the cells themselves. In fact, the division of lymphocytes on a microscope slide depends entirely on the presence or absence of a chemical agent, and, if it is present, on its strength and on its diffusion into the cell.

In our opinion, judging from the mitotic figures

which have been induced in cells, mitosis should not be described as the phenomenon of *nuclear* division. It is part of the cycle of cell-division, and the whole of the cell-protoplasm takes part in it. The Altmann's granules form the chromosomes, the nucleolus forms the centrosomes, and the nucleus forms the spindle. The protoplasm of the cytoplasm and cell-wall also reproduces itself and divides during mitosis.

The active principle in the stain which causes mitosis in lymphocytes is a constituent of the azur dye. This dye also contains a substance which kills the protoplasm, and having done this it will, if in sufficient concentration, cause that protoplasm to stain scarlet. Mitosis occurs by the action of the active principle on the chromosome granules; cell death occurs by the action of the poison on the centrosomes. So far the active principle has proved to be inseparable from the poison in the anilin dye.

Up to this stage in the researches the only substance we had found which would induce divisions in lymphocytes was this anilin dye; but its action was augmented by atropine and an extract of dead hæmal gland. Atropine augments its action five-fold if it is absorbed in suitable strength, in which case it may induce asymmetrical mitosis. Neither atropine, in no matter what strength, nor extract of dead hæmal gland in the strength of 100 per cent will by themselves induce mitotic figures in lymphocytes.

Great care must be exercised in the practice of inducing the mitotic figures in lymphocytes. The jelly must be accurately prepared, but it is better to

allow it to be deficient in alkali by a unit or so at first. A film is made with some fresh blood spread on it and incubated for ten minutes. The temperature of 37° C. must be accurate; many failures have resulted owing to the neglect of regulation of the incubator temperature. If the chromosome-granules of the lymphocytes are unstained, a drop or two of alkali solution is added to the jelly and a fresh film is tried. Soon the right alkalinity will be obtained to induce early mitosis in the ten minutes; and now if more alkali is very carefully added to the jelly and another film made, later stages of mitosis will be induced. It is instructive to proceed farther and once more add alkali, when the cells will be killed too quickly, and only very early stages will be seen in the ten minutes, for there has not been time for late phases to occur before the cells have died. If more alkali is again added, owing to rapid death the cells will appear quite at rest, as if there had been no agent to cause cell-division in the jelly at all. But the granules and nucleoli will be deeply stained, and the polymorphonuclear cells will probably all be burst and achromatic.

When first we showed the mitotic figures to some of our friends we received some adverse criticisms. It is always possible to induce mitosis in lymphocytes, but it is not always possible, at a few minutes' notice, to find figures which resemble the diagrammatic drawings of mitosis in the cells of plants and the lower animals as given in the text-books on cytology. However, when a convincing figure did appear, the nature of the chromosomes, the spindle, and the centrosomes were

immediately appreciated. We, of course, maintained that the divisions were induced by a specific chemical substance contained in the stain, pointing out that lymphocytes had never been seen to divide before, and that mitosis will only occur in them if they absorb a certain quantity of the substance. But our friends "one and all began to make excuse." Some said that the divisions were in the nature of a death-struggle; they pointed out—a fact which we admitted—that death always was premature, and it usually occurred during the act of mitosis. We explained the cause of death, but still the suggestion of the "death-struggle" was maintained by some in the absence of proof against it.

Others suggested that the divisions were entirely artificial and not at all like the natural method of cell-proliferation, although they had never seen the latter. We admitted the fact that at this stage of our researches we could only induce divisions in lymphocytes, and we could only do this with an entirely artificial anilin dye; but still we found it difficult to appreciate why a cell should go out of its way to divide by an entirely abnormal process. We suggested that we thought that if a cell was going to divide at all, it would try to do so by the normal process to which it was accustomed. But the suggestion that the mitotic divisions were "freaks" remained to be disproved.

It appeared to us a remarkable thing that a cell should try to reproduce itself by cell-division in a death-struggle; it seemed such a futile thing for it to do. Moreover, other stains—such as ordinary

methylen blue—do not induce divisions like azur, and yet they kill the cells by staining the chromatin of the nucleolus-centrosome. Why should only the latter dye cause cell-division; presumably both would cause death-struggles? Moreover, we have often killed cells by prussic acid and nitro-benzol, but no division occurred and nothing resembling a death-struggle. Again, in connection with the experiment given in this chapter in which it was ascertained that the stage reached in a single act of mitosis varies directly with the quantity of the chemical substance absorbed, it appeared to us that if these mitotic figures induced in lymphocytes were in the nature of death-struggles, a cell once it had been started in its act of mitosis would continue that act until it was complete. But as the experiments showed, they did not do so, for when the diffusion of the chemical agent was arrested the mitosis ceased even in its early stages.

These suggestions were worthy of consideration, and the only way to disprove them was to continue the investigations. It appeared to us reasonable to suppose that other cells, besides lymphocytes, would possibly respond by dividing to the chemical auxetic, and we also considered it possible that other chemical agents existed which would induce divisions. Lymphocytes responded in such a constant manner, and always required a definite quantity of the substance that we thought it possible that there might be something similar to it in the body which would cause their proliferation. The question was, What was this substance and where was it contained? Was it associated



with the cell-proliferation of healing? Lymphocytes proliferate in healing, especially in chronic healing, and chronic healing is a forerunner of cancer. Still leucocytes proliferate even more than lymphocytes in healing, but we had never up to this point, nor had any one else, ever seen a leucocyte divide. If the agent in the azur dye was analogous to a chemical substance in the body which caused the cell-proliferation of healing, it ought, strictly speaking, to cause the multiplication of polymorphonuclear leucocytes as well as lymphocytes. But so far it had not done so.

All these points were carefully considered at this stage, and they urged us to make further researches. Whether we were right or wrong in supposing that there might be a chemical auxetic in the body which caused proliferation of cells in a manner similar to the agent contained in the azur dye, we had made one step in causing one class of human cells to divide on the microscope stage by means of a chemical agent.

## CHAPTER XII

THE "EXPERIMENTAL TEN MINUTES"—DIVISIONS INDUCED IN THE SO-CALLED POLYMORPHONUCLEAR LEUCOCYTES—METHOD FOR COUNTING THE NUMBER OF GRANULES CONTAINED IN EOSINOPHILE LEUCOCYTES, AND THE REDUCTION OF THIS NUMBER IN THE CELLS OF CANCER PATIENTS.

THE reduced number of chromosomes exhibited in lymphocytes when they are forced to divide in ten minutes by increasing the diffusion of the stain into them with alkali reminded us forcibly that the cells were undergoing mitosis under the stress of experimental conditions. The somatic number of chromosomes of human cells is 32; and since lymphocytes will, if their divisions are delayed, divide by the somatic number, it appeared to us that the normal time occupied by the division of the cells in the body is probably much longer than the ten minutes allowed to them on the microscope slide. It was appreciated that it would be better if we could delay the diffusion of the stain into the cells to such an extent as always to produce somatic divisions. But unfortunately herein we met with a difficulty which has not yet been overcome. As we have pointed out, death has been delayed

for twenty minutes while mitosis has been induced, but this experiment has only been followed by success on very few occasions. For general practical purposes it must be remembered that whatever is done in the way of attempting to induce divisions in cells when they are resting on a jelly-film, this must be done in ten minutes. If the diffusion of the chemical agent is delayed beyond this time, except in very few instances, the cells will refuse to divide at all, simply because they die.

The reason for this is a question of vitality, which brings us back to the disadvantages of *in-vitro* experimentation. All cells of the body lose vitality gradually after they have been shed. White blood-corpuscles will live in citrate solution for two or three days at the room temperature, but they lose vitality all the time. As already pointed out, there is no known medium in which blood-cells will live and thrive, and in the best medium at our disposal they merely exist for this short period. When cells are resting on a jelly-film, however, they are not even in the best available medium; but at present we are bound to employ the jelly method, for we have not succeeded in inducing divisions in any other way. The reason for this is two-fold: firstly, because substances can be made to diffuse into individual cells more quickly if the cells are *pressed* into the jelly which contains them; and, secondly, we think that lymphocytes prefer to be at rest when they divide, for we cannot induce divisions with the cells floating in a solution, although we have tried to do so many times in solutions which have contained the necessary constituents.

At present there is nothing for it but to induce divisions with the cells spread out on jelly under a cover-glass; and it must be remembered throughout that these conditions are most detrimental to the cells. Pressed in this way into the jelly by means of a cover-glass, which to living cells must be proportionately of enormous weight, leucocytes and lymphocytes will not live more than about three-quarters of an hour. Although they will exist for this time, and although amoeboid movements may be excited in them during greater part of it, it is obvious that the cells are in reality dying slowly all the time. Since the ease with which one can induce divisions in lymphocytes varies directly with the vitality of the cells, it is clear that whatever is done to induce mitosis must be done quickly, and by practical experiment it has been found best to observe the general rule that, when one attempts to induce cells to divide on the microscope slide one must so arrange the jelly-film that the cells will be in the act of mitosis within ten minutes. This is a serious disadvantage appertaining to *in-vitro* experimentation, which cannot so far be overcome, and it is important to remember it throughout. The cells are labouring under abnormal difficulties which modify one's deductions from the facts seen; and since this important point will frequently have to be considered, it is convenient to standardise these detrimental conditions and designate them the "experimental ten minutes."

Two corollaries depend on the "experimental ten minutes." Since the induction of a division in a cell depends on the diffusion into it of a certain quantity of

a chemical agent, and since the quantity of the agent required must increase with the rapidity at which we wish mitosis to occur, it is obvious that a greater concentration of the chemical agent will be required to induce a division in the "experimental ten minutes" than would be required to make a cell reproduce itself if it were resting in its normal surroundings, where it might take a much longer time in its division.

The second corollary is that if the jelly on which the cells are resting contains a saturated solution of a given substance which is diffusing into the cells to the utmost in ten minutes, and if that substance does not induce divisions in the "experimental ten minutes," it does not prove that that substance will not within the body, with the cells in their natural surroundings, cause them to proliferate.

It was a matter of concern to us that the azur dye did not make the polymorphonuclear leucocytes (fig. 76) divide. So far only lymphocytes responded. If the contention was correct that the dye contains a specific agent which was possibly analogous to some similar agent in the body which causes proliferation of lymphocytes, it appeared reasonable to expect that some similar agent, if not an identical one, would also cause divisions in leucocytes; for the latter cells always proliferate together with, and to a greater extent than, lymphocytes during the process of healing. So far, however, we had not seen anything resembling a division in a polymorphonuclear leucocyte. It must be admitted that we had no idea as to what a leuco-



FIG. 76.—A resting polymorphonuclear leucocyte. Its granules are stained but not its nucleus. The cell was alive.

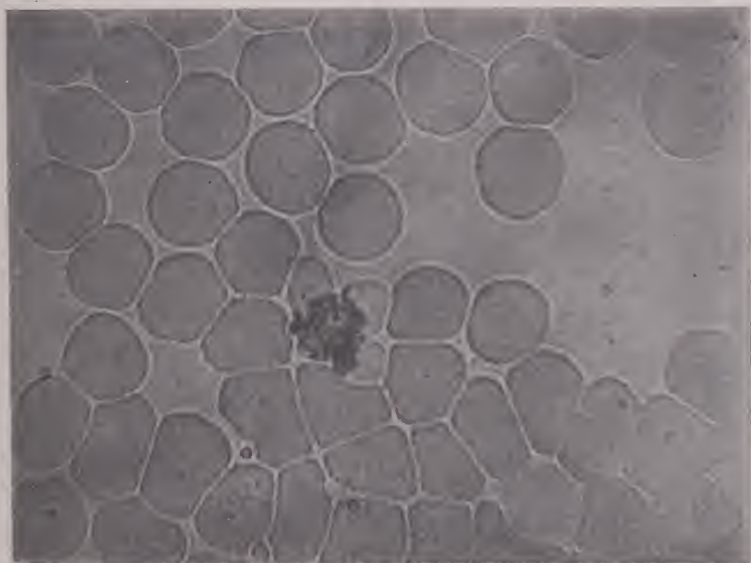


FIG. 77.—A basophile leucocyte in the act of cell-division. The granules of the cell are in the centre. The lobes of the nucleus are at the poles of the cell which is dividing into three.





cyte would look like when it divided, for no one had ever seen a division in a leucocyte. These peculiar cells are large, and easily examined. They differ from all other cells in that they contain a polylobed nucleus, and it was very difficult to imagine how mitosis would occur in such a cell. Speculations have been made from time to time to the effect that these cells divide by pluripolar mitosis. Each lobe of the nucleus is said to undergo a mitosis of its own, and that the chromatin within the lobe forms up into chromosomes. This would mean that a cell with five lobes to its nucleus would divide into ten cells. Such a speculation makes no allowance for the Altmann's granules, which attain a large size in these cells, or for the filaments which unite the several lobes of the nucleus. Since the cytological process of mitosis in lymphocytes was so different to what was expected, we were prepared to see the speculation disproved; but in spite of this it must be admitted that when at last the divisions of leucocytes were seen the arrangement of their cytological elements came rather as a revelation.

Jelly-films were made which contained greater strengths of the azur dye, extracted from polychrome stain in the way which we have described. The possibility of divisions being induced in leucocytes was considered to be an event which would be seen before long; but when it was first seen it, like the first mitosis in lymphocytes, was not recognized or appreciated. The increased quantity of azur dye was added to the jelly in reality to see what the effect of

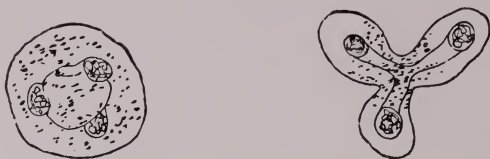
excess of it on lymphocytes might be. On one occasion a "basophile" leucocyte was found lying on the jelly with its granules arranged in rows, and forming a sort of radiating pattern. Moreover, the granules were in the centre of the cell, which is an unusual position for them, and there were clear spaces outside them which evidently contained the lobes of the nuclei, although the latter were not stained, as it is very difficult to stain the nuclei of basophile leucocytes *in vitro*. The condition had not been seen before, but it was passed over, for at the time lymphocytes were being sought for. Some days afterwards another basophile cell was seen in a similar condition, and then it was more carefully observed. The lobes of the nucleus of this cell could just be made out, and they were external to the granules. The cell-wall itself was indented in three places, so that the leucocyte looked like the propeller of a steamship. The granules were deeply stained and turning black (fig. 89), which sometimes occurs *in vitro* in the stained granules of basophile cells; and they were again arranged in indefinite lines or rows. It was this arrangement of the granules which specially arrested attention. The Altmann's granules of lymphocytes form up into rows to form the chromosomes, and it looked as if something of some similar nature was happening on this occasion in a leucocyte.

This curious condition of the basophile leucocyte seemed to have occurred in response to the excess of azur dye. Still more of it was therefore added to some coefficient jelly which also contained atropine, polychrome dye, and extract, with the idea of de-

liberately producing this condition of the basophile leucocytes. As a matter of fact, the jelly contained 0.6 cc. of the azur dye, and its index of diffusion was now arranged for the coefficient of diffusion of the basophile leucocytes, which is the same as that of the ordinary neutrophile leucocyte.

After removal from the incubator at the end of ten minutes, it was seen that the lobes of the nuclei of the neutrophile polymorphonuclear leucocytes were just staining a faint blue colour, and—there could be no question about it—nearly every leucocyte in the specimen was in the act of division. Neutrophile, basophile (fig. 77), and those eosinophile (fig. 78) leucocytes which were not ruptured were undergoing the act of reproduction on the jelly-film. They were dead owing to the staining of the lobes of their nuclei, but the lines of demarcation between the individual daughter cells could be distinctly seen. The cytological procedure by which these cells divide is identical in all varieties of leucocyte. As in lymphocytes, the Altmann's granules were formed into rows, and presumably they are analogous to chromosomes; the rows of granules become arranged into indefinite lines radiating outwards from the dividing-point, which is in the centre of the cell. Running down through the centre of the mass of granules, the filament which unites the lobes of the nuclei evidently forms a basis, analogous to the spindle of other cells, to which the chromosomes are attached; and at the poles of this filament, or spindle, the so-called lobes of the nuclei appeared. It was then immediately appreciated that

these bodies are in reality the centrosomes of the cells.



If a leucocyte has two lobes to its nucleus it will divide into two cells; if it has three lobes it will divide into three cells, and so on. It will thus be seen that when these cells proliferate each daughter cell will have one centrosome until that centrosome itself divides and assumes the appearance of being polylobed. Further, a tissue made up of such daughter cells would be described as consisting of “mononuclear cells.” The chromatin-staining lobes within the leucocytes are therefore not nuclei but centrosomes, and the so-called Altmann’s granules, which have been variously surmised to be collections of food or secretion, are the elements of the chromosomes themselves.<sup>1</sup> As in lymphocytes so in leucocytes, the chromosomes are *outside* the nucleus. Divisions have been induced in hundreds of leucocytes, and the procedure is always the same in all of them (figs. 79–86).

Now, the increased quantity of the azur dye contained in the jelly did not improve the mitosis induced in the lymphocytes; in fact, it seemed too strong for

<sup>1</sup> Professor Sherrington has a specimen of an eosinophile leucocyte of a cat in which the individual granules are elongated and almost rod-shaped. We have also seen elongated granules in these cells in human blood.

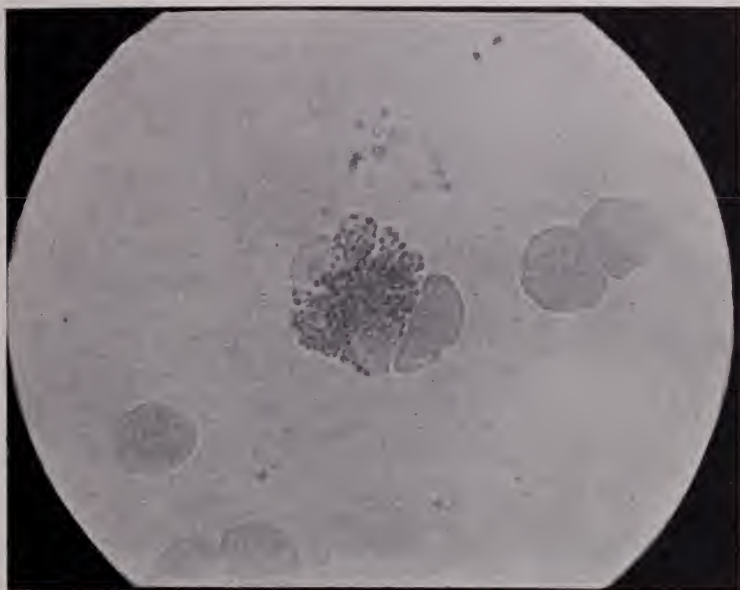


FIG. 78.—An eosinophile leucocyte in the earliest stage of division. The granules are arranged in lines radiating outwards from the centre of the cell. The lobes of the nucleus were at the poles.



FIG. 79.—Early stage of division of a neutrophile leucocyte.







FIG. 80.—A dividing leucocyte.



FIG. 81.—A dividing leucocyte.



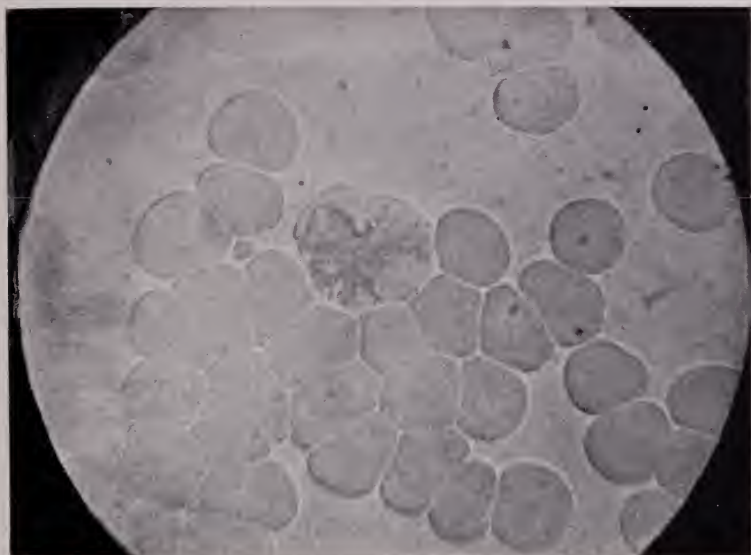


FIG. 81A.—Division of a leucocyte. The linear arrangement of the granules could be well seen.



FIG. 82.—A dividing leucocyte.





FIG. 83.—A dividing leucocyte.



FIG. 84.—A dividing leucocyte.







FIG. 85.—A dividing leucocyte.



FIG. 86.—A dividing leucocyte.



them, as only early stages of mitosis were seen in them. Hence it became apparent that the auxetic constituent of the aniline dye which induces divisions in lymphocytes also does the same thing with leucocytes; but it evidently requires more of it, *ceteris paribus*, to induce a division in a leucocyte than in a lymphocyte. The coefficient of diffusion of the lymphocyte is higher than that of the leucocyte if the staining of the nucleus is the moment by which it is determined; but so far as inducing divisions is concerned the coefficient of lymphocytes seems to be lower, for they require less of the chemical agent than do leucocytes.

The divisions of reproduction had now been induced in both leucocytes and lymphocytes by an artificial chemical agent. These cells are the ones which proliferate when a tissue is damaged, and it is by their multiplication that the healing of an injury takes place, and it must be borne in mind that cancer, with its increased malignant proliferation, is intimately associated with chronic healing. Judging by the divisions induced in these white blood-corpuscles it appeared that their reproduction takes place in a cycle which depends on some chemical substance absorbed by them. The cycle consists apparently of the division of the centrosomes, division of the chromosomes, and the division of the cell. Whether there is a "resting stage" in the strict sense of the term, we are not in a position to state, for we do not know how long a time is occupied in the division of the centrosome. If a cell is absorbing the agent which causes it to divide, presumably the cycle of mitosis is going on in direct proportion to the

amount of the agent absorbed. The division of the centrosome seems to be part of this cycle, but how long this part takes we do not know. Hence it appears possible that what is commonly known as the resting stage is in reality the time occupied by the division of the centrosome.

The method of division of leucocytes and lymphocytes is so constant that we thought it was reasonable to expect that the proliferation of healing would be ultimately proved to take place by a similar process, and that if so there must be produced in an injured tissue some chemical substance very similar in its effects to that contained in azur dye. Up to this time, however, we had not succeeded in inducing divisions at all with any substance which we could call a "natural" substance. There is nothing in the body that we know of at all like the aniline dye. It was true that an extract of dead hæmal gland augmented the action of the aniline dye; but it would not induce divisions by itself. Extracts of tissues other than hæmal gland were tried, made in the same strength—namely, 100 per cent—and it was found that suprarenal glands of sheep augmented the action of the stain in inducing divisions even better than hæmal gland, and several extracts, such as those of muscle and liver, did the same, but to a lesser degree. In spite of the augmenting action of all these extracts, however, none of them alone in the strength tried would induce divisions either in lymphocytes or leucocytes in the experimental ten minutes.

This inability to cause cell-division by entirely "natural" substances, such as the extracts named, was

believed, after mature consideration, to be due to the fact that we had not tried extracts in sufficient strength for cells to respond to them under the detrimental circumstances of the "experimental ten minutes." It has already been pointed out as a corollary to these circumstances that if a cell refuses to respond to a given substance by not dividing in the experimental ten minutes, it does not prove that that substance does not actually contain an active principle for inducing cell-division. We therefore considered the advisability of concentrating the extracts, and then trying them again by themselves.

In the first instance this concentration process took some little time. At this stage of our researches we were unaware that the active augmenting principle contained in the extracts was "thermostable" and would resist boiling, and in consequence, at the outset, we evaporated the extracts at the room temperature, which was a most tedious process. Moreover, during this slow concentration of the extracts it was necessary to test them from time to time to see if their augmenting action was impaired at all with keeping.

In the meantime other points were considered. It is well known that cancer-cells frequently are seen to be dividing with a reduced number of chromosomes. As we have already stated, we believe that a reduction in the number of chromosomes is due to increased proflificity in cells; and this being the case, it seemed probable that there might be some increase in or augmentation of the cause of proliferation of the cells of cancerous growths. Further, if this is the case, the



augmenting substance might appear in the peripheral circulation. It has already been pointed out in Chapter VIII. that cancer plasma excites amœboid movements in leucocytes, and that alkaloids also excite these movements. Since the alkaloid atropine augments the action of azur stain in inducing divisions, it was thought possible that the exciter of amœboid movements found in cancer plasma might be in the nature of an alkaloid possibly derived from the neighbourhood of the growth. Now, atropine and stain together cause white blood-cells to extrude granules of chromatin, a phenomenon which we erroneously called "flagellation" (see Chapter X.), and this extrusion had also been observed in cells which have been subjected to cancerous plasma.<sup>1</sup> The suggestion followed that the cells might be extruding their granules deliberately in response, not only the artificial combination of stain and alkaloid, but also to some possibly similar combination derived from the malignant growth. Moreover, since in both the artificial and the natural circumstances, cells appear to divide with a reduced number of chromosomes, and since the granules form the chromosomes, it was surmised that the extrusion might be part of the process of reduction. It must be remembered that our experimentation left us convinced that the divisions of lymphocytes and leucocytes occur just as the stain is combining with the chromosome-granules; and as the extrusion of the granules—which has been seen by others as well as ourselves—seems to be a deliberate

<sup>1</sup> "The Flagellation of Lymphocytes in the Presence of Excitants both Artificial and Cancerous," by H. C. Ross and C. J. Macalister, *British Medical Journal*, January 16, 1909.

action on the part of the cells, we went so far as to theorise that the cells might be discarding their granules in order to prevent some of the combination of the "auxetic" and their granules, and so delay their proliferation to some extent.

This theory led to the suggestion that we should try to count the number of granules contained in the blood-cells of cancer patients, with a view to see if they were reduced in number in that disease. The blood of cancer patients seems to contain a body which excites amœboid movements and the extrusion of granules, and, therefore, the blood-cells themselves as well as the cells composing the growth might also have a reduced number of the granules which form their chromosomes.

We must admit that these suggestions were based on slender grounds of evidence, and it was appreciated that to count the number of granules of the leucocytes of cancer patients would require considerable work, especially as many control experiments would have to be made, for we did not even know the normal number of granules in healthy persons' cells. Still, it was very necessary to try to find out whether the clue on which we were engaged was in any way correct, and it was realised that in order to make the counts it would be necessary to examine a large number of samples of blood-cells from many patients and from normal and other persons—a procedure which had not yet been done by this *in-vitro* method. Hence no matter how far-fetched it appeared at first sight for us to count the granules of blood-cells in cancer patients, I thought that an endeavour to do so would be justified, and I devised the following technique for doing so.

It was seen from the outset that it would be quite impossible to count the number of granules contained in lymphocytes, and the same could be said of those of the common neutrophile leucocyte (fig. 76). But it is possible to do so in the so-called eosinophile cells (fig. 87). These cells have large granules, which stain a deep scarlet with the polychrome dye, and therefore these cells were chosen for this series of experiments, especially as they are fairly common (2 to 4 per cent).

Three difficulties presented themselves in arranging a technique for counting the number of eosinophile granules:

1. To the novice the basophile cell is sometimes very difficult to distinguish by *in-vitro* staining from the eosinophile cell, and mistakes seriously modify the results. If specimens of each class of cell are seen lying side by side (fig. 88) there is no difficulty in distinguishing them, the eosinophile cell being much the larger, although there is very little difference between the size of their granules. But in spite of the fact that the cells rarely are thus found lying side by side, with a little experience they can be readily distinguished; the granules of the basophile cell are more discrete, and the lobes of its nucleus will practically never stain by this *in-vitro* method.

2. A living leucocyte is spherical in shape, and it usually appears with its granules heaped one on top of another, rendering it impossible to count them accurately.

3. If one attempts to count through the microscope a group of granules not arranged in any definite order,

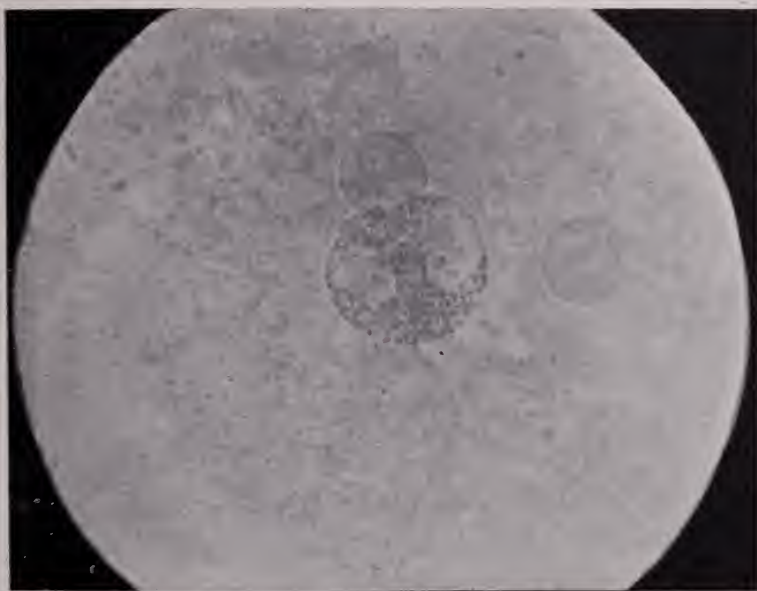


FIG. 87.—An eosinophile leucocyte with its granules stained.



one is apt to count the same granule more than once, and it is easy to lose one's place—in which case it becomes necessary to begin all over again. On looking at the cells through the microscope, the granules appear as though they might with care be counted, and it is most inviting to attempt to do this and to rely on it; but on testing this rough-and-ready method we have found that it usually involves an error of nearly 50 per cent. No estimate whatever can be made of the number of granules contained in a cell by merely looking at it through the microscope, no matter what magnification is used.

Obviously the granules must be stained, and then it is necessary: (1) to distinguish readily between an eosinophile and a basophile leucocyte; (2) to kill the cells, and then to burst them so as to cause their stained granules to rest discretely side by side in one plane and not on top of one another; (3) to magnify the image of the ruptured cell in such a way that one can "tick off" each granule with a pencil on paper as it is counted, so as to avoid counting the same granule twice over.

By the following procedure the staining, killing, differentiation, and bursting can be readily accomplished. In order to magnify the image of the ruptured cell so as to count its granules and to "tick them off," it is necessary to obtain a photomicrograph negative of it, and then to project the photographed image on to a paper screen with an optical lantern, when the image of each granule can be marked off on the paper with a pencil.



It is necessary to employ the photomicrographic apparatus which I have already described, and the photographs must be taken with as little delay as possible after the cells have been ruptured. Unfixed cells may rapidly become achromatic after death, and, in the case of a ruptured cell, the loss of stain may occur with great rapidity.

The blood of the person to be examined is drawn into a capillary tube and there mixed with an equal volume of citrate solution. At the room temperature this solution will keep the cells alive for some days; but when it is intended to count the granules of the eosinophile leucocytes, it is better to examine the blood as fresh as possible.

A jelly is prepared thus: To a tube containing 5 cc. of coefficient jelly add 4 units of Unna's polychrome stain, 7 units of the 5-per-cent alkali solution, and, instead of making the contents of the tube up to a total of 10 cc. with water, 3.9 cc. of a molten 2-per-cent *solution of agar* in water is used. The last solution contains agar in order to make the jelly exceptionally firm, so that the ultimate bursting of the cells can be facilitated. The jelly is melted and boiled and a drop of it run on to a slide, where it is allowed to set. A drop of the citrated blood is then placed on a cover-glass, which is inverted and allowed to fall flat on the film in the usual way. The slide is then placed in the 37° C. incubator for three minutes exactly. When examined microscopically it should be seen that the nuclei of the eosinophile leucocytes are just staining scarlet, showing that death is occurring; the granules



FIG. 88.—A field containing a neutrophile, an eosinophile, and a basophile leucocyte. The upper cell is the neutrophile and the lower one the basophile cell. All the cells are ruptured, but their granules are stained.



FIG. 89.—A basophile leucocyte whose stained granules have been turned black by heat.



of the cells should be deeply stained. If the nuclei are not yet stained, a little more alkali must be added to the jelly and a fresh specimen made. If the cells are achromatic or disorganised, or if the nuclei of the neutrophile cells are deeply stained, the jelly is too alkaline, and a little acid solution must be added to it. But if the coefficient jelly and other solutions are correct, the nuclei of the eosinophile cells will just be staining.

Using a  $\frac{1}{6}$ -inch or equivalent objective the specimen is searched until a suitable eosinophile cell is found. If a cell is distorted or hemmed in by red cells, it is necessary to pass it over and find another.

If there is any doubt as to whether a cell is an eosinophile or basophile one, the slide is removed from the mechanical stage in such a way that on returning it to the microscope the same field can be focused again. The slide is then again incubated for three minutes, but at 47° C. On examination of the cell, if it is an eosinophile leucocyte, its granules will still appear scarlet; but if it is a basophile cell, its stained chromosome granules will have turned black<sup>1</sup> (fig. 89). With a little experience of the method of staining, however, the difference between the classes of cell can be detected without this procedure of incubation at 47° C., which is apt to cause premature rupture and achromasia.

The next step is to burst the cell. The photomicrographic apparatus being ready on its slide above the observer's head, the immersion objective is "turned

<sup>1</sup> These granules may turn black at 37° C. We have no explanation to offer of this phenomenon.

on" and focused, and the cell is brought into the centre of the field. Watching it through the eye-piece, keeping one hand on the fine adjustment, the cover-glass, which of course is resting on the jelly-film, is gently struck (tapped) with a glass rod held in the other hand. At each tap the cells are seen to be jerked out of the field, but, provided the taps are not too forcible, the eosinophile cell can easily be followed by using the mechanical stage. It is usually necessary to strike the cover-glass two or three times, and generally at the third blow the eosinophile is seen to totter and then burst, scattering its stained granules about on the surface of the jelly in the field of the microscope. This is a trick, of course, which was devised by one of us (J. W. C.), and with a little practice rupture can nearly always be assured.

When a cell ruptures on this jelly—which contains salts—its nucleus loses its stain instantly, but at the room temperature the granules do not usually become achromatic for some little time. On the other hand, in some instances they may become unstained in a few moments, and for this reason, in order to secure the photographic negative, speed is now required. The ruptured cell is placed in the centre of the field with the mechanical stage; the working eye-piece is removed from the microscope, the camera is allowed to slide down the wooden slide, and its projecting eye-piece, which is already attached to it by means of a flexible velvet collar, is inserted into the draw-tube of the microscope. By the simple movement of swing-

ing the microscope mirror on its gimbals out of the focal axis, the working 32-c.p. gas-light is changed to the water-cooled ray of light from the 1-amp. Nernst lamp. The image of the ruptured cell will then be seen on the ground-glass screen at the back of the camera, where it can be rapidly focused.

The special precautions regarding the focusing with this method have already been described, but it should be remembered that in order to be able to count the number of the granules in the ruptured cell it is most important to obtain as perfect a negative (figs. 90, 91) as possible.

If the photography has been accomplished quickly, the camera may be pushed up out of the way, the microscope mirror replaced, and the specimen may be searched for more eosinophile leucocytes.

To count the number of granules contained in a ruptured cell, the negative must—after it has been developed and dried in the usual way—be placed in an optical lantern, and the image of the ruptured leucocyte projected on to a screen which has a sheet of white paper pinned in front of it. One stands close in front of the screen and counts the granules, each of which will now appear about the size of a shilling-piece, and the image of each granule can be “ticked off” with a pencil on the paper (figs. 92, 93). It is thus impossible to count any granule twice over, and an accurate enumeration can be made.

Such is the technique. By it there have been counted 38,759 granules from 235 cells from 96 persons,



22 of whom were suffering from undoubted<sup>1</sup> cancer,<sup>2</sup> and 47 of whom apparently were not. Of the latter, which for convenience will be called the "control" cases, some were "healthy" and others were suffering from various diseases (hospital patients). We did not count the granules in each of the 235 negatives as the latter were obtained, but the plates were developed and then put away until a hundred or more had collected. The name of the person from whom the blood had been taken was entered into a book with the age, sex, disease (if any), and other details. The negatives were numbered consecutively, and the numbers corresponded with similar ones in the book against the names of the persons from whom the cells had been derived. The samples of blood were taken from persons, cancerous or otherwise, as they came into hospital, and therefore, without referring to the book, a number on a negative gave no indication from whom the cell it depicted was derived. With three exceptions, the samples of blood were collected and photographed by one of us, who kept the book in his laboratory. The counting was done by another, who had no idea to whom the numbers on the negatives referred.

The only possible source of error is in the counting. Some of the negatives were not quite perfect, and some of the granules appeared blurred; hence there may be a small error in some of the numbers, but it cannot be very important judging by the uniformity of the averages.

<sup>1</sup> Determined either by such clinical manifestations as recurrence or metastasis, or by pathological examinations.

<sup>2</sup> Carcinoma.

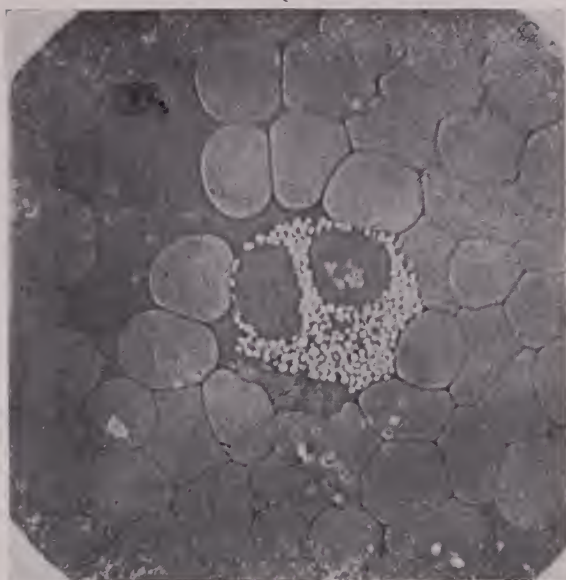


FIG. 90.—One of the negatives of a ruptured eosinophile leucocyte (negative No. 52).



FIG. 91.—One of the negatives of a ruptured eosinophile leucocyte (negative No. 54).





FIG. 92.—Counting the granules. The image of the ruptured cell depicted on negative No. 52 is projected on to a sheet of white paper pinned on to a screen.



FIG. 93.—Counting the granules of negative No. 54.



At first only two cells were taken from each person, but since it was found that there was frequently a wide variation in the number of granules contained in individual cells, this number was afterwards increased to five. Averages were then struck, and the tables given in Appendix I. give the number of leucocytes examined and the persons from whom they were derived, together with the number of granules contained in the largest and smallest cells from each person. From these averages it will be seen that the sex makes practically no difference in the average number of granules contained in the cells; but more experiments will be needed before the same can be said about age. The averages can, in the first place, be divided into two groups, male and female. Each of these groups can be subdivided into two, viz. control persons (healthy and diseases other than cancer), and cancer persons. Neglecting fractions, the average number of granules in the cells appear thus:

Number of persons	Average number of granules in cells of			
	males		females	
47 controls . . . . .	168	. . .	168	
22 cancer . . . . .	159	. . .	161	

Thus, between normal<sup>1</sup> males and females there is no difference, and between carcinoma males and females there is very little difference; but the number of granules in cancer-cells is well below the normal in the averages of both males and females, and it would appear from this that the number of granules contained in the cells of cancer patients is actually reduced.

<sup>1</sup> Control cases (normal, and diseases other than cancer).



The table and its summary supply further details. As was expected, the reduction is not very large, but the striking point is that, in addition to the total cancer averages being below the normal, a subdivision into such groups as male and female demonstrates that the reduction in cancer is again present in *both* groups.

Every individual case of cancer in the category does not, by any means, have a reduction in the average number of granules contained in the cells, and it will be seen that many of the individual controls showed a reduction; but when one comes to deal with comparatively large numbers, the reduction in carcinoma is demonstrated. It must be remembered that in everybody there is a great variation in the actual number of granules contained in individual cells; and when sampling say five cells from a person, one may by chance hit upon five larger or five smaller cells. Obviously, therefore, it is only by the observation of many cells from large numbers of persons that one can reduce to a minimum the "error of random sampling." We think, however, that the enumeration of the granules contained in 235 cells, from 22 cancer patients and 47 controls, diminishes this error to such an extent that the results are fairly trustworthy. At the same time, it must be remembered that in experimentation of this nature the error of random sampling can never be altogether eliminated, and therefore the reliability of the averages depends entirely on the extent of this error among the cells which have been photographed.

Among the control cases three cases of sarcoma are included. In all of them there was no apparent reduction, and the same can be said of another case tested recently. But the number of cells is too small to form any conclusion from, and more cases will be required.

## CHAPTER XIII

THE AUXETIC ACTION OF CANCER SERUM—THE INDUCED DIVISIONS OF GRANULAR RED CELLS—THE AUXETIC ACTION OF “THE REMAINS OF DEAD TISSUES,” AND ITS AUGMENTATION BY ATROPINE AND THE PRODUCTS OF PUTREFACTION—THE ISOLATION OF THE AUXETICS KREATIN AND XANTHIN—DISCOVERY OF THE CAUSE OF THE CELL-PROLIFERATION OF HEALING

COUNTING the granules of eosinophile leucocytes from cancer patients, therefore, seemed to us to show that the clue on which we were working was to some extent correct. Judging from the comparison between the number of granules contained in the cells of cancer patients and those of other people, there appears to be a reduction in cancer, and this reduction presumably is due to the presence in the blood of some agent which causes more proliferation than normal. It has been pointed out that increased prolificity owing to excessive absorption of a chemical agent makes cells divide by a reduced number of chromosomes as seen in carcinoma cells; and now apparently other cells, such as the eosinophile leucocytes, have in that disease a slightly reduced

number of chromosome granules. But this digression from the main researches also taught us that other facts were to be learnt from the comparison of samples of peripheral blood from twenty-two cancer patients and forty-seven "others." Never before had systematic examination of blood from such groups of persons been made by the *in-vitro* staining of their cells, and it was soon noticed that in the samples of cancer blood the actual number of eosinophile leucocytes was reduced; in fact, four cases could not be included in our category, because, even after repeated examination of many samples of their blood, no eosinophile leucocytes could be found; and in all the other cases, with the exception of three, there was an undoubted reduction in the number of eosinophile cells. In the three exceptions there appeared to be an eosinophilia.

In some cases of carcinoma, also, there was a large-lymphocytosis, especially in the advanced cases. But this is by no means an absolute rule, and, moreover, a large-lymphocytosis was fairly common among the control specimens.

But a still more important point was observed. We have already shown how a mixture of azur dye and atropine causes excitation of amœboid movements in leucocytes and lymphocytes, then the discard of granules (flagellation), and lastly augmented cell-division; also that an agent has been detected in the plasma of carcinoma patients which induces the first two—*i.e.* excitation of amœboid movements and the discard of granules. We have just shown that the granules in certain leucocytes in cancer

patients are reduced in number. The inference is that this reduction is made in response to the same agent which causes the excitation and discard of granules. The important point is that while engaged in these blood examinations the fact became apparent that this agent in cancer plasma (presumably it is the same agent) will help to induce cell-division.

The large lymphocyte requires a considerable quantity of stain, extract, or atropine before it will be induced to divide in the "experimental ten minutes." In the technique, described in the last chapter, for counting the granules of eosinophile leucocytes the jelly employed contains only 4 units of polychrome dye, the efficiency of which for inducing divisions is infinitesimal (the jelly containing no extract of dead tissues or atropine). Yet in the examination of the blood of three of the cases of carcinoma some of the large lymphocytes showed well-marked stages of early mitosis, whereas this result could not be obtained in any of the controls. It is clear, therefore, that the cells in these three cases were inclined to divide before they were ever placed on the jelly, and the trifling assistance which they received from the 4 units of the polychrome dye caused them to show well-marked mitotic figures (figs. 62, 64), whereas the large lymphocytes in all the control specimens, made under exactly the same conditions, remained at rest.

Moreover, in two other cancer patients (both cancer of the stomach), owing to anæmia, many granular red cells were seen in their blood. On its being examined on jelly which contained azur dye, extract, and atropine,



FIG. 94.—A dividing red cell from a cancer patient.

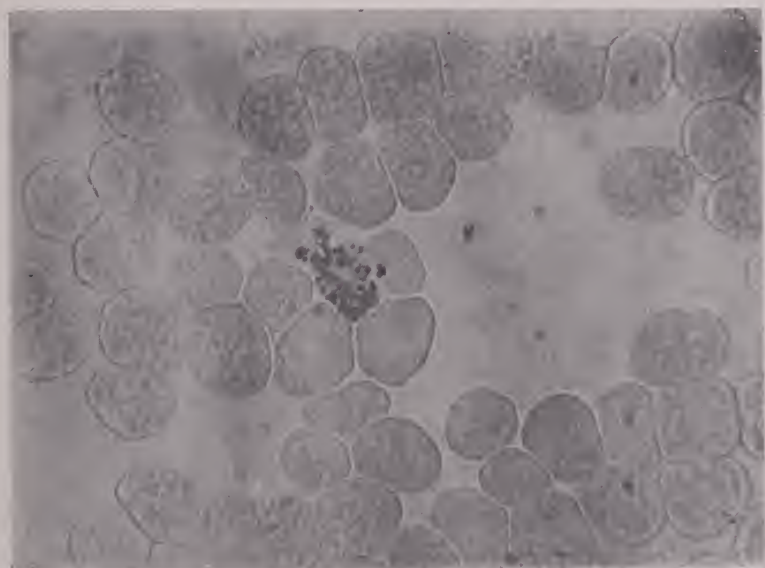


FIG. 95.—A dividing red cell from a cancer patient. The granules seem to be arranged in an indefinite figure.





amitotic<sup>1</sup> divisions (figs. 94, 95) were induced in these granular red cells. The granular red cells of normal and other persons have never hitherto been seen to make any attempt to divide on auxetic jelly or any jelly, and hence it appears that these cells from these cancer patients are also more prone to divide than those of other people.

Since it has been shown that the reproduction, certainly of lymphocytes and leucocytes, and possibly of other cells, depends (on the microscope slide) on the quantity of an auxetic absorbed by them, it is reasonable to suggest that the plasma of these cancer patients contained some such agent which caused this inclination to divide on the part of the large lymphocytes and red cells. Presumably this is the same agent which had been previously found to cause excitation of amœboid movements, and the discard of granules for the combination of stain and atropine will also do this as well as cause augmented divisions.

It is interesting to note that it is only the red cells which have granules which can be induced to divide, for it bears out the theory that the auxetic contains a specific agent which induces cell-division by acting on cell-granules.

We may now return to the study of the extracts. It may be remembered that we had only succeeded in inducing divisions in lymphocytes and leucocytes with the artificial azur dye. Extracts of several

<sup>1</sup> We are uncertain whether some of the granular red cells were not dividing mitotically (fig. 95), as their granules appeared to be arranged in an indefinite figure.

dead tissues, especially that of suprarenal gland, in the strength of 100 per cent, would augment the action of the azur dye, but they would not in themselves induce divisions or even the early stages of mitosis in the experimental ten minutes. We had therefore made arrangements to concentrate these extracts so as to see if they would, if used in greater strength, induce divisions by themselves. At first it was thought better not to boil down the extracts for fear that the boiling might spoil the substance which augmented the action of the dye. The extracts were therefore placed in test-tubes, which were lightly plugged and put aside in the laboratory. As already mentioned, it was necessary to test these extracts from time to time to see whether they might become more effective as concentration occurred. When they were originally made they were sterile, because it may be remembered that they had been kept at 60° C. for twelve hours after filtration. Repeated examination of some of the tubes, however, caused them to become infected, and in consequence putrefaction set in in those tubes. After they had all been kept for three weeks it was noticed that the augmenting action of the contents of one of the infected tubes of suprarenal extract seemed to be increased. One cc., or even a few drops, of this extract, if added to the azur dye and made up in a jelly, caused advanced mitosis in lymphocytes, whereas with the other sterile tubes it seemed to require about the usual quantity of extract to augment the action of the dye. It was particularly noticed that this tube which contained so efficient an extract had

been examined on several occasions, and owing to the infection of its contents the latter was in a foul-smelling condition. The increased augmentation when this decomposed extract was used was so remarkable that we decided to try its action by itself without any azur or other stain.

The jelly was made up thus: To 5 cc. of coefficient jelly 3 cc. of the putrid extract, and 0.8 cc. of 5-per-cent solution of sodium bicarbonate (8 units of alkali) were added. The alkali was present in order to cause the contents of the jelly to diffuse into the cells. The jelly was made up to a total of 10 cc. with 1.2 cc. of water. In order to prevent coagulation of the extract a film was prepared from the jelly in the following way: The coefficient jelly was melted and boiled, and it was only as it cooled that the extract was added, the film being made immediately before the jelly had set in the test-tube. Fresh blood from the finger was spread on the jelly in the usual manner under a cover-glass. After incubation for ten minutes, an examination showed that some of the lymphocytes appeared to be in an early stage of mitosis. Now, we could not be very certain about this point, because no stain was present and consequently the chromosomes were unstained and almost invisible. If mitotic divisions are sometimes difficult to see in stained specimens, they are much more difficult to distinguish when no stain is employed. Still, the cells looked rather as if they were attempting to divide (fig. 96).

A fresh jelly was made, but it contained 1 cc. of alkali solution instead of 0.8 cc.; and now there was

no doubt about it—this extract did actually induce mitotic figures in lymphocytes in the experimental ten minutes (figs. 97, 98). No azur stain, atropine, or other “augmenter” was added; the decomposed suprarenal extract induced mitosis by itself.

Of course we thought at first that this result was due to the concentration of the extract; but this thought was soon dispelled by trying some of the other tubes which had been kept alongside of the ones which had so often been examined, the contents of which had decomposed. The sterile extract contained in these tubes would not induce divisions by themselves. Moreover, at the temperature of the laboratory, since they were kept in plugged tubes, the extracts did not evaporate very fast, and it was appreciated that they could not be so very concentrated. Some of the effective putrid extract, therefore, had water added to it, so that it was again made up to its original strength of 100 per cent. It was then made up in a jelly as before, and to our astonishment again it induced divisions in lymphocytes; and what is more important, it induced the asymmetrical one-sided mitosis in many instances (fig. 99).

A series of control experiments was then made. Jellies which contained only the salts sodium citrate, sodium chloride, and the 1 cc. of alkali were first tried, and no divisions could be seen. Then yet another series of experiments with fresh extract of suprarenal gland was made, once more without result, and so it was ultimately proved that it was

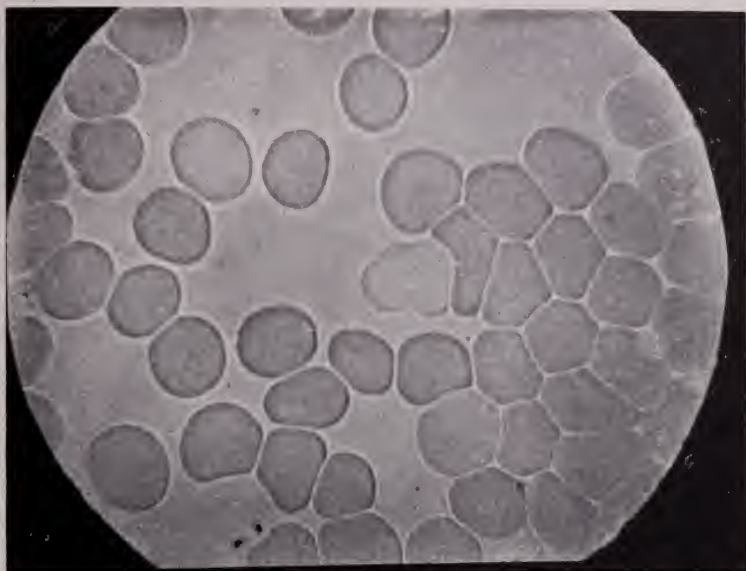


FIG. 96.—Very early stage of mitosis in a lymphocyte induced by decomposed extract of suprarenal gland. No stain.



FIG. 97.—Mitosis of a lymphocyte induced by decomposed suprarenal extract. No stain.





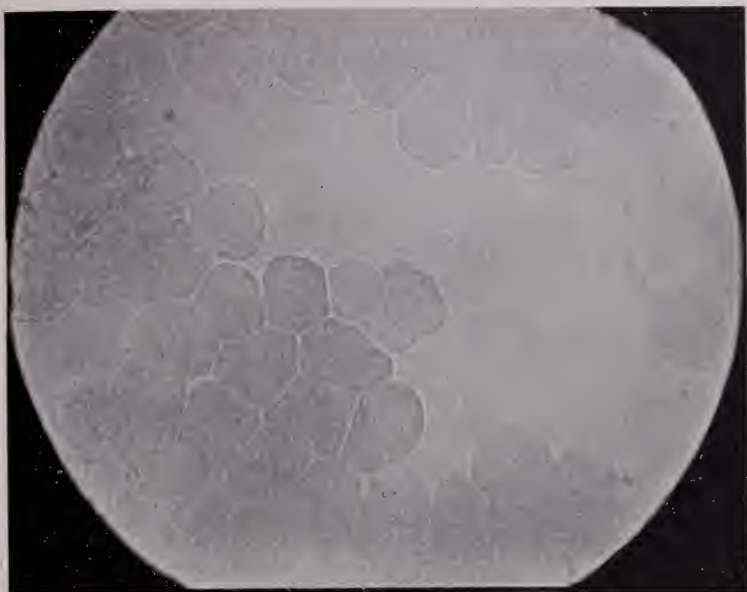


FIG. 98.—Mitosis induced in a lymphocyte by decomposed extract. No stain.



FIG. 99.—Asymmetrical division induced by decomposed extract. No stain or atropine is present.



unquestionably due to the putrefaction that this one tube of extract induced divisions in the experimental ten minutes.

Now, this fact required very careful consideration. A 100-per-cent solution of extract would not in itself induce divisions in lymphocytes unless it was putrid. When it is fresh this extract is not effective in the experimental ten minutes. It appeared probable that the extract does in itself contain some substance which causes cell-division, but in the strength of the extract of 100 per cent this substance is not present in sufficient quantity for it to induce divisions in the experimental ten minutes unless the whole extract is putrid. The first thing to do was to concentrate the extract and see if *this* theory was right. It was appreciated that the concentration process at the room temperature was a most unsatisfactory procedure, for if the extracts were tightly plugged they did not evaporate down, but if they were left open they became putrid. One of the jellies which induced divisions by virtue of the putrid extract was therefore boiled and tried again. Still it induced divisions in lymphocytes. It was submitted to prolonged boiling, and yet it was effective. So it was proved that the substance which it contained which caused cell-division was thermostable. We can boil these extracts with impunity, and their auxetic action is not impaired. Hence we made some fresh extract of suprarenal gland and evaporated it down to dryness by boiling. It is, when dry, a hygroscopic brown mass which is readily soluble in water. One

hundred grammes of sheep's suprarenal glands yields about 4 grammes of dry extract.

A series of jellies were prepared which contained 0.8 cc. of alkali solution (8 units), variable quantities of solutions of the extract, and they were always made up to the total of 10 cc. with water. At first a 5-per-cent solution of the extract was made; and it was found that if the jelly contained 1 cc. of this extract, very early divisions can be induced in lymphocytes. With 2 cc. later stages of mitosis will appear (figs. 100, 101); and if instead of the 5-per-cent solution a 10-per-cent one is made, even more marked divisions can be induced by this fresh extract alone in the experimental ten minutes. The best jelly to make in order to cause suprarenal extract to induce divisions in lymphocytes in the ten minutes is: 5 cc. of coefficient jelly, 1 cc. of alkali solution, 2 cc. of a 10-per-cent solution of dried suprarenal extract, and 2 cc. of water. By means of this jelly advanced mitotic figures can be induced in lymphocytes.

So it was proved, therefore, that this extract of dead suprarenal gland contains a substance which will cause the divisions of lymphocytes. A fresh jelly was then prepared the same as the last one, except that it had added to it four more units of alkali solution. Now, as we anticipated, the polynuclear leucocytes also divided on the microscope slide (fig. 102).

But the question was then asked, How was it that the original extract, although it was not strong enough to induce divisions by itself in ten minutes, did become effective when it was decomposed by putrefaction? It

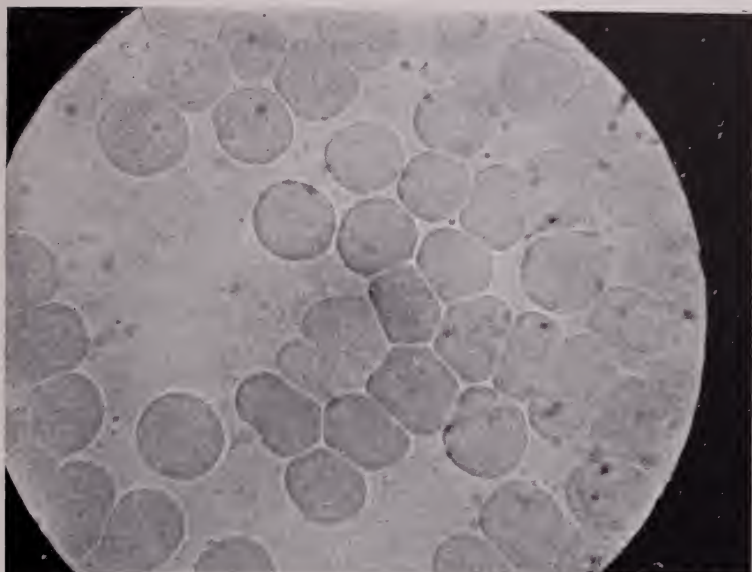


FIG. 100.—Mitosis induced by fresh extract of suprarenal gland. No stain or augmentor present.

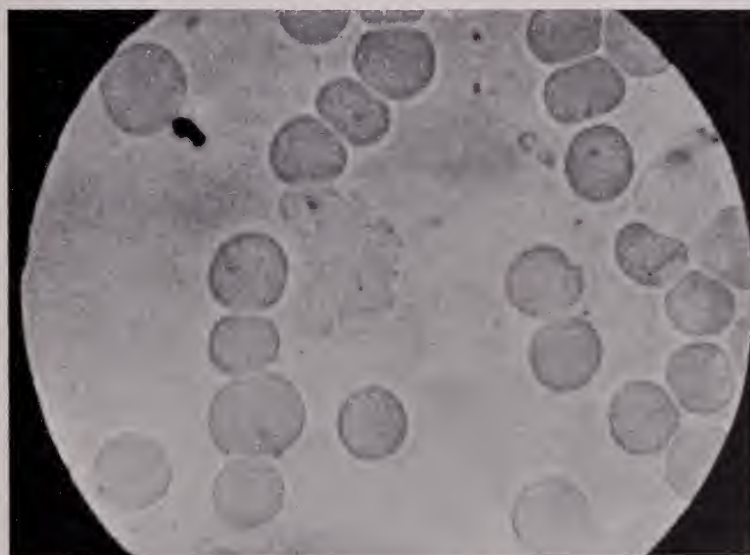


FIG. 101.—Mitosis induced by fresh suprarenal extract. No stain is present.





was evident that the first extracts which we tried were not strong enough to induce divisions in the experimental ten minutes. If they became putrid, however, they apparently were.<sup>1</sup> The putrid solution was again tried, and again the asymmetrical divisions were seen. Now, these asymmetrical divisions are frequently induced by azur dye when it is augmented by atropine, and therefore we thought that it might be possible that the putrefaction of the extract might produce in it an augmenting substance which acted like the atropine.

Fresh suprarenal extract was then made, and after it had been dried it was redissolved in water. It was made up in a 10-per-cent solution, and various quantities of it were added to jellies which contained 1 cc. of alkali solution (10 units), and it also had added to it 0.7 of a 1-per-cent solution of atropine sulphate. It was now found that the atropine augmented the action of the suprarenal extract five-fold, in the same way as it augmented the action of the azur dye—that is to say, with suprarenal extract by itself, and *no* atropine, the 10 cc. of jelly, if it contains alkali to the extent of 10 units, must contain at least 0.05 gramme of dried suprarenal extract before the earliest sign of cell-division can be induced in ten minutes. To obtain well-marked divisions the jelly should contain 0.2 gramme of the extract.

If atropine is added, however, in the strength of

<sup>1</sup> Some 100-per-cent suprarenal extract has been purposely allowed to become infected, when it induced divisions in lymphocytes (figs. 103, 104). Control tubes of extract not so infected had not this action.

0.007 gramme of atropine sulphate to the 10 cc. of jelly which has 10 units of alkali, divisions in lymphocytes can be induced if the jelly also contains no more than 0.01 gramme of dried suprarenal extract. Once more we tried to induce divisions with the alkaloid by itself, but failed; and yet it augmented the action of the extract five-fold. In addition to this augmentation it induced asymmetrical mitoses (fig. 105).

To recapitulate: Extract of suprarenal gland of certain strength will induce by itself mitotic divisions in lymphocytes; and if more of it is made to diffuse into cells, it will also cause leucocytes to divide. If a lower concentration is tried, however, it will not induce divisions in the experimental ten minutes unless (1) it has become putrid, (2) its action is augmented by atropine. In both the latter circumstances asymmetrical mitosis may be seen.

Other extracts of dead tissues were then tried; but they would not, by themselves, induce divisions in the experimental ten minutes. Realising that this might be due to the detrimental experimental conditions (corollary 2), we tried them again with atropine to augment their action. Now, as surmised, all the extracts of dead tissues which we tried induced divisions in lymphocytes on the microscope slide. To induce divisions in polymorphonuclear leucocytes with them is much more difficult, as atropine does not appear to augment their action so much with these cells.

The following table gives the strengths of the various extracts which, with 1 cc. of alkali (10 units) and 0.007 gramme of atropine sulphate, will induce

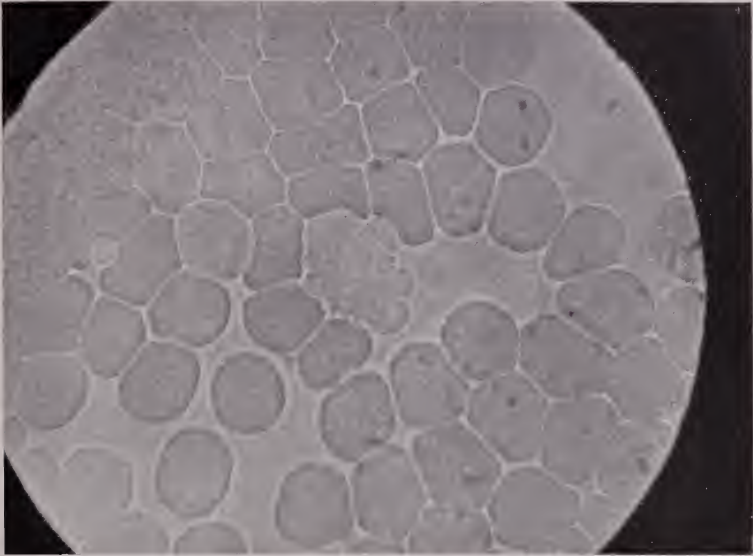


FIG. 102.—A dividing polymorphonuclear leucocyte induced by suprarenal extract alone. No stain.

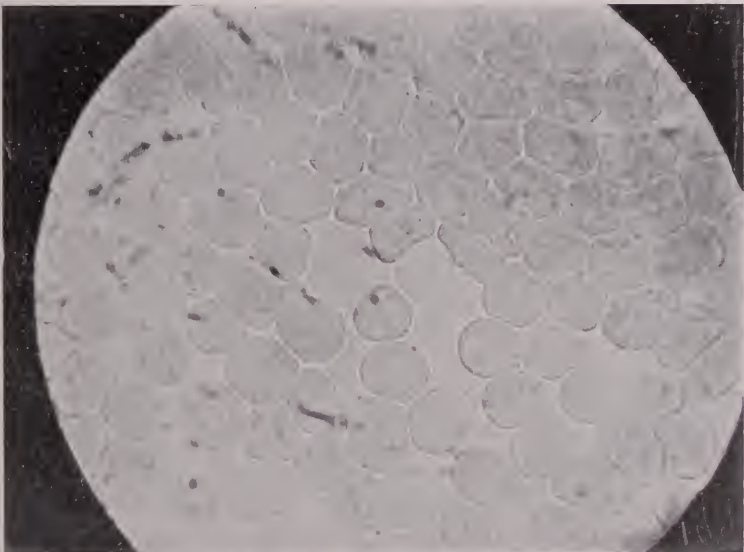


FIG. 103.—Mitosis induced in a lymphocyte by suprarenal extract which had purposely been allowed to become putrid. No stain.



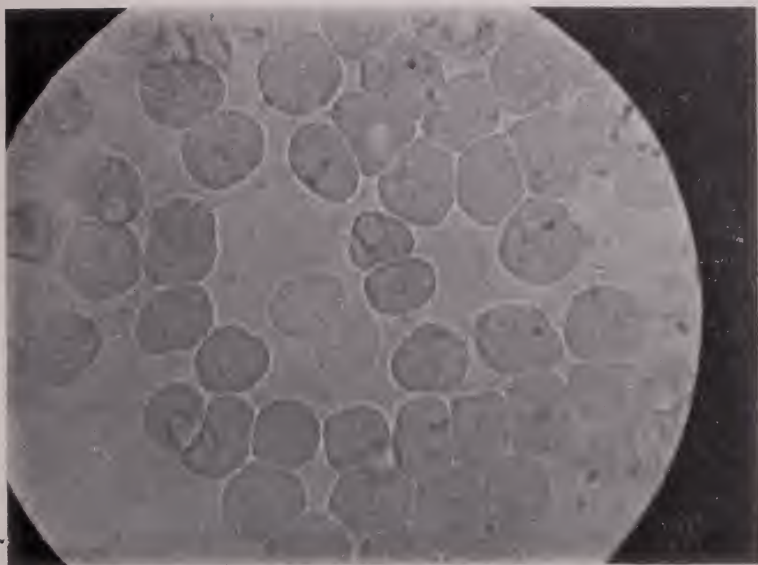


FIG. 104.—Mitosis induced in a lymphocyte by suprarenal extract which had purposely been allowed to become putrid. No stain.

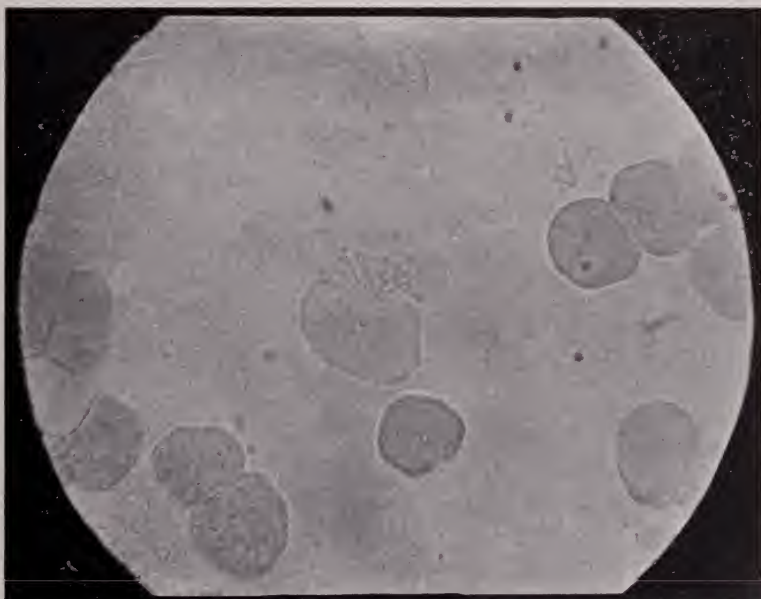


FIG. 105.—Asymmetrical mitosis induced by suprarenal extract augmented by atropine. No stain.





divisions in lymphocytes in the experimental ten minutes.

			Amount to be contained in the 10 cc. of jelly.		
Dried extract of	Testis	.	.	.	0.025 gramme
"	"	"	Pancreas	.	0.025 "
"	"	"	Muscle	.	0.025 "
"	"	"	Spleen	.	0.01 "
"	"	"	Liver	.	0.002 "

Experimentally all these extracts were employed in a 5-per-cent solution. Divisions in lymphocytes were induced with the first three by adding 0.5 cc. of the solution to the 5 cc. of coefficient jelly, together with 0.7 cc. of 1-per-cent solution of atropine sulphate, 1 cc. of the 5-per-cent solution of sodium bicarbonate, and made up to a total of 10 cc. with 2.8 cc. of water. The jellies were boiled and films made from them in the usual way.

It is obvious, therefore, that all the extracts contain some substance or substances which cause cell-division in lymphocytes and in leucocytes. To induce these divisions on the microscope slide in the experimental ten minutes, it is necessary to augment the action of the extracts with atropine. Suprarenal extract, however, evidently containing more of the active substance than the others, will induce divisions without any augmenting substance. Putrefaction will augment the power of the extracts like the alkaloid, and it was presumed that this putrefaction had this effect through the presence of the alkaloids of putrefaction. This point, however, was not investigated till afterwards, as we were immediately concerned in finding out if possible what the agents were in these extracts of dead tissues which cause the division of white blood-corpuscles.

There were two ways in which we might attempt to isolate this active principle from the extracts. We might analyse them and try the different substances one by one. These analyses had, however, often been done before, and it was considered better, in the first instance, to try the well-known constituents of these extracts to see if they would induce cell-division before we undertook to analyse the extracts ourselves.

We need not detail the vicissitudes of this research, which occupied a long time. The constituents of the extracts of the body are well known. It may be remembered that the active principle in the extracts is evidently thermostable, and remains in solution after most of the proteins have been precipitated by heat. We tried certain salts, and other substances, and we have also tried urea, and at last *kreatin* ( $C_4H_9N_3O_2$ ) was found to be a substance which will induce divisions in lymphocytes (fig. 106) and leucocytes (fig. 107). Kreatinin ( $C_4H_7N_3O$ ) is not effective in the experimental ten minutes; but *xanthin* ( $C_5H_4N_4O_2$ ) is if its action is augmented by atropine.

The following table gives the strengths of kreatin and xanthin required to be contained in the 10 cc. of jelly in order to induce divisions in lymphocytes in the experimental ten minutes, no atropine being employed, but the jellies contained 1 cc. (10 units) of alkali solution.

Kreatin.			
0.02 gramme	.	.	No mitosis seen.
0.04     "	.	.	Early mitosis.
0.75     "	.	.	Well-advanced divisions.



FIG. 106.—Mitosis induced in a lymphocyte by kreatin. No stain or extract.



FIG. 107.—Division in a leucocyte induced by kreatin. No stain or extract



With atropine:

Kreatin.				
0.005 gramme	.	.	.	No mitosis.
0.01     “	.	.	.	Mitosis.
0.02     “	.	.	.	Well-advanced divisions.

Employing xanthin the presence of atropine is necessary:

Xanthin.				
0.002 gramme	.	.	.	Early mitosis.

If the jelly contained saturated solution<sup>1</sup> of xanthin, well-marked figures were seen.

We had now succeeded in inducing the reproduction of leucocytes and lymphocytes—first by the aniline dye azur, then by a substance contained in the extract of suprarenal gland, and by our experiments we were able to infer that this substance is contained in the extracts of other dead tissues. It has just been shown that this inference is correct, because cell-division can be induced by the crystalline extractive kreatin, which is a constituent of the remains of all dead tissues. So far, of course, we had only induced divisions with these substances *in vitro*; but, as already pointed out, the cells are under very detrimental conditions while being experimented with, and it is more than probable that, if they will divide in response to these substances *in vitro*, they will more readily respond to them *in vivo*.

Healing is caused by the proliferation of leucocytes and lymphocytes, and, judging from the *in-vitro* experimentation, this proliferation is evidently induced by kreatin and

<sup>1</sup> Xanthin is sparingly soluble.



xanthin. Hitherto it has been generally supposed that the cell-proliferation of healing is due to some inherent propensity on the part of the cells to divide; but now it is clear, from *in-vitro* experimentation, that these cells divide when they absorb a definite quantity of a chemical agent, and two of these auxetics are kreatin and xanthin, which are contained in the remains of dead tissues. When a tissue is damaged anywhere, cell-death is occasioned, and the dead cells liquefy. The products of this death have as constituents the extractives kreatin and xanthin, and we know that the neighbouring living cells must absorb the liquefied remains of their dead neighbours, for it has been shown that the diffusion of substances into living cells is a physical process over which the cells themselves can exercise no control. When a tissue is damaged, therefore, the direct result of that damage will be to make the neighbouring living cells reproduce themselves in response to kreatin and xanthin, and bring about the cell-proliferation of healing.

Here, then, is the solution of the first part of our problem. We now know the nature of the physiological cause of the cell-proliferation of healing, and we submit that this knowledge reveals a fresh vista in pathology.

But it must not be supposed that kreatin and xanthin are the only agents contained in the remains of dead tissues which cause cell-reproduction. They are two of the active principles which we have so far succeeded in isolating. It is probable that there are others; in fact, we know that there must be. Supra-renal extract will induce divisions very readily, and the

amount required to do so is so small that the kreatin and xanthin which it contains will not account for the divisions it induces. These bodies are amido-acids, and we think that the  $\text{NH}_2$  group of the molecules may be responsible for the auxetic action. In this respect it is interesting to note that alkaloids, which augment the action of auxetics, are compound ammonias; but it must be remembered that we have never yet been able to induce a division with an alkaloid by itself, although we have tried literally hundreds of times.

In the next chapter we shall show that there is another great and very important source of the "causes of the cell-proliferation of healing" contained in a substance we call "globin," a histone derived from hæmoglobin.

## CHAPTER XIV

### THE AUXETIC ACTION OF GLOBIN

THE fact that *in-vitro* experimentation has shown that cell-division is directly caused by certain constituents of the soluble remains of dead tissues made us consider the possibility that there might be other sources of these or similar agents. It was remembered how frequently old chronic ulcers, when they heal, leave the tissue pigmented, and it was considered possible that this pigmentation might in some way be associated with the healing process and its cell-proliferation. The pigment in ulcers is supposed to be derived from hæmoglobin.

Melanotic sarcoma is generally accredited to be the most prolific of all malignant growths. It is characterised by the pigmented cells of which it is composed. We have not been able to obtain a case of melanotic sarcoma, for such cases are rather rare, but it is generally the case that the pigment is contained in the cytoplasm of the malignant cells. One of the

commonest sites of melanotic sarcoma is in the choroid coat of the eye, where the cells are normally pigmented. The pigment of these cells is called melanin, and it is supposed to be derived from hemoglobin.

Professor Ronald Ross suggested that some experiments might be made with auxetics on the malaria parasite, and in one case a "crescent" was apparently made to flagellate prematurely with a jelly containing azur dye, extract, and atropine, although repetitions of the same experiment were not successful. Still, the consideration of the life-history of the malaria parasite has been—as it turns out—germane to our researches. The parasite enters the body from the mosquito as a minute unpigmented amœbula, which straightway enters a red blood-corpuscle. While in the red cell it gradually becomes pigmented, and it proliferates by exporulation. The daughter parasites have no pigment until they enter fresh red cells, when in their turn they become pigmented and ultimately proliferate again.

There is the so-called sexual form of the cycle, however, which probably does not proliferate within the body. The crescent or gametocyte only proliferates after the blood containing it has been shed. The crescent is also deeply pigmented; and it is a most interesting point to remember that when the crescent stage of the parasite is reached, the red cell appears to be depleted of hæmoglobin, and merely surrounds the parasite as an empty cell. The parasite, when it has reached the crescent stage, has apparently

devoured all the hæmoglobin; the hæmatin derived from the hæmoglobin has collected in the parasite as a pigment known as melanin; and the parasite will no longer proliferate until the blood is shed. *If* the blood is shed, however, whether it is shed on to a microscope slide or into the stomach of the mosquito, the parasite again becomes prolific almost immediately, and flagellation occurs.

Now, when blood is shed, no matter how it is shed, whether it be on a microscope slide or into the stomach of the mosquito, hæmoglobin must be set free, for the red corpuscle is a very delicate cell, and many of them must be ruptured when any injury occurs in a tissue. The question therefore arises, Does hæmoglobin have any function in inducing the proliferation of the malaria parasite? From circumstantial evidence it would appear that it does, for so long as the parasite is absorbing hæmoglobin from the red cell in which it lives, so long will it continue to proliferate by exporulation; but when it has finished the contents of the cell, proliferation ceases until more hæmoglobin can be absorbed by it when the blood is shed.

In the malaria parasite, in the cells of melanotic sarcoma, and in the neighbourhood of old healing ulcers the hæmoglobin is evidently decomposed because the hæmatin collects as insoluble pigment.

Hæmoglobin is fairly soluble, but when it is decomposed into hæmatin and globin the hæmatin is insoluble in water except in the presence of dilute alkalis. Globin is readily soluble. Hence it cannot

be the hæmatin part of the hæmoglobin molecule which has any function in causing proliferation; it must be the globin part if it is either of them.

In the first instance we tried the effect of hæmoglobin on blood-cells. Jellies were made which contained 1 cc. (10 units) of alkali solution, and after they had been boiled various quantities of a saturated solution of crystalline hæmoglobin were added before the jellies cooled too much for them to set on a slide. But hæmoglobin never induced divisions in lymphocytes or leucocytes in the experimental ten minutes. Nor did it excite amœboid movements in them.

We next made a saturated solution of hæmoglobin and then boiled it, thereby decomposing it and precipitating the hæmatin. The filtrate is a straw-coloured liquid when it is dilute. It was evaporated down by prolonged boiling, and at the saturation point, which is about 4 per cent, the solution becomes a deep red colour. On evaporation to dryness, a sticky residue remained. Very little is known about globin. For years it was thought to be a globulin, but this has been shown not to be the case. Globin is a histone—a protein which is not precipitated by boiling. In the dry state it is a glutinous mass of a deep brick-red colour, and it has a characteristic sweet smell something like licorice. If it is very dry, globin can be ground into a brown powder. It is at all times extremely hygroscopic, and therefore if it is not kept in solution it must be placed either in a desiccator or in sealed tubes. If it is kept in solution and exposed to the air, it rapidly decomposes owing to putrefaction,



and gives off a foul smell, reminding one of that of the alkaloid neurine.

Jellies were made which contained various strengths of globin, and, of course, certain quantities of alkali solution were also added. It was found that globin by itself would never induce divisions in lymphocytes in the experimental ten minutes, so we tried it again with the addition to the jellies of 0.7 per cent of atropine sulphate, and then globin induced divisions in lymphocytes (figs. 108, 109). This is the best strength to employ: In 10 cc. of jelly containing 10 units of alkali and 0.007 gramme of atropine there should also be 0.0025 gramme of globin. The best divisions are obtained with 0.025 gramme of globin; but if the content of it exceeds 0.05 gramme, the cells appear to be poisoned, because they shrivel up and frequently burst.

Some globin in solution (1 per cent) was allowed to putrefy for a fortnight, and, like extracts of dead tissues, it was then found that its action was so augmented that it also would (in the strength of 0.005—or better 0.01 gramme—in the 10 cc. of jelly) induce divisions by itself (without atropine) in the experimental ten minutes (fig. 110).

When putrefaction occurs in a solution of globin a precipitate falls, and yet it is now more effective in inducing divisions than it was before. It is clear, therefore, that it is not actually globin which induces divisions, but it is some constituent of it which is effective. Putrefaction decomposes globin, and the active agent plus some augmenting substances are produced.

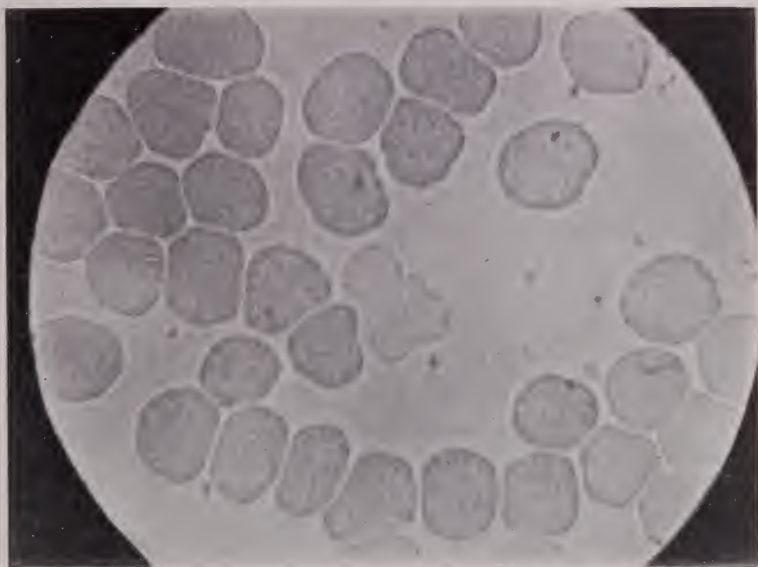


FIG. 108.—Mitosis in a lymphocyte induced by globin augmented by atropine. No stain, extract, or kreatin.



FIG. 109.—Asymmetrical mitosis induced by globin augmented by atropine. No stain, extract, or kreatin.



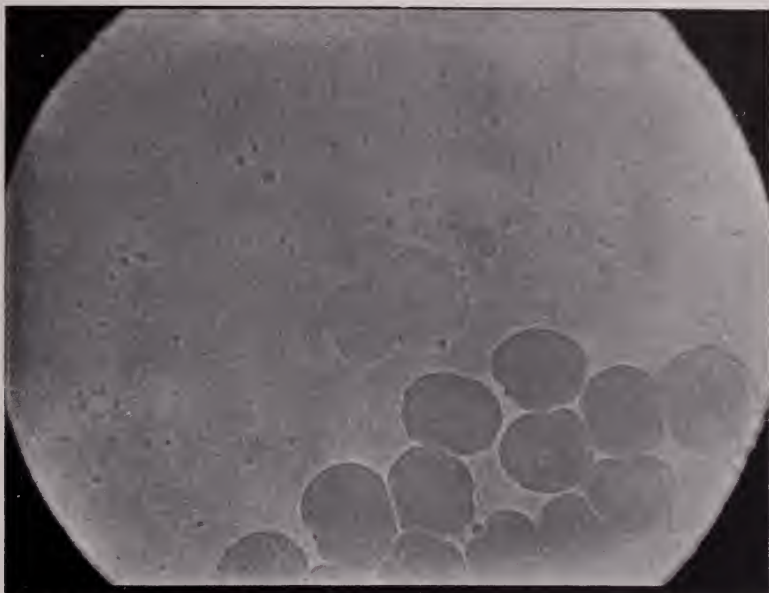


FIG. 110.—Mitosis induced in a lymphocyte by means of decomposed globin solution. No stain, extract, kreatin, or atropine.



It must be understood that if the jelly on which the cells are resting contains 0.02 gramme, or more, of globin, the red cells become distorted and the white cells are killed without divisions being induced in them.

We think that it should be mentioned that it is quite within the realms of possibility that the malaria parasite proliferated in response to the active agent contained in globin; but although we have tried a few experiments to endeavour to prove the point, we have not succeeded in determining it. Malarial crescents frequently flagellate in any case within ten minutes of their being shed; and although we have mixed the shed blood containing them with citrated solutions of globin, it has been impossible for us to satisfy ourselves that the flagellation has been accelerated by its action. In the cases of malaria at our disposal there have not been a very large number of parasites in the blood, and time was lost during the experiments in finding them. Hence we cannot speak definitely on this point, but it was the consideration of the life-history of the malaria parasite which was the chief factor which led us to investigate the auxetic property of globin; and there is no doubt whatever that globin contains some auxetic, although it is not so powerful as that contained in suprarenal extract.

Globin contains no kreatin so far as we can ascertain, and the solution of globin which we have used is free of hæmatin, as proved by spectroscopic examination, and there are only traces of lipochrome. What the exact nature of the auxetic substance contained in



globin is we do not know, but possibly it is allied in some way to the molecules of kreatin and xanthin. It should also be remembered that we do not know what the substance is in the azur dye which induces divisions. We think that they will not be difficult to isolate; but we ourselves do not feel competent to undertake chemical analyses of this nature.

## CHAPTER XV

THE PROOF THAT THE REMAINS OF DEAD TISSUES AND GLOBIN CONTAIN THE CAUSES OF THE CELL-PROLIFERATION OF HEALING AND OTHER CELL-REPRODUCTION—EXPERIMENTATION *in vivo* CONFIRMS *in-vitro* OBSERVATIONS—THE CAUSE OF BENIGN TUMOURS

THE foregoing experiments show that some of the causes of human cell-division are now known. On the stage of the microscope white corpuscles can be made to undergo the stages of cell-division in direct response to certain chemical agents, two of which have been isolated, and which can be employed in crystalline form to induce cell-division. What is far more important, however, is the source whence these chemical substances are derived. They are contained in the soluble remains of dead tissues. Another source of the cause of cell-division is in globin, which is derived from the decomposition of hæmoglobin.

It should be remembered that so far the experimentation has been confined to testing the action of the active substances and the sources of them on individual

cells which have been removed for the purpose from the body; and, as already pointed out, the cells in this *in-vitro* experimentation are not by any means in conditions similar to the natural ones under which they normally exist. Still there is no question whatever that the cells do divide in response to these agents; and if they will do so under detrimental experimental conditions, it is obvious that they will be far more likely to divide and respond to the same agents; in their normal conditions. The agents we know do not exist in the body, and therefore it is practically a certainty that these substances will cause proliferation there. For reasons already given, on the microscope slide one cannot induce more than one generation of cells by chemical agents, because premature death cannot be prevented; but in the body the premature death need not necessarily occur, for its cause is absent, and hence, provided the causes of cell-division are being constantly supplied to cells, generation after generation must be produced.

On the microscope slide cells will not divide, so far as can be seen, unless they absorb definite quantities of the agents which cause cell-division. We do not say that there are no other substances which cause cell-division besides those which have been mentioned—in fact, we know that there must be others; but what we think is now becoming evident is the fact that cells will not divide at all unless they receive some chemical agent which makes them do so. That is to say, we think that there is strong evidence in support of the view that cell-division in the body is entirely caused by

chemical agents; and if these agents are not present, there will be no cell-division.

In the case of leucocytes. For nearly a century and a half these cells have been observed in the blood. Every doctor and student of medicine must have seen them alive repeatedly, and yet not a single person had ever seen them divide. Now, however, if one makes them absorb certain chemical agents the cells divide immediately; and what is more, we have shown that the rapidity of onset and the time occupied by each division varies directly with the quantity of the substances absorbed by the cells. Cell-division appears to be a physical phenomenon which can be measured in the case of each cell in proportions of grammes of the chemical auxetics absorbed by them. We have shown how it can be set down as a simple mathematical equation. It must be admitted that in spite of the fact that blood-cells have not been seen to divide without an auxetic, there is no actual proof that a cell cannot divide without one. It has yet to be proved that human leucocytes have no inherent power to multiply "when they feel so inclined," but it is a remarkable thing that no single leucocyte, out of the many millions which have been seen by men, should ever have developed this inclination during nearly a century and a half. On the other hand, we know that if we cut our fingers and so produce the remains of dead tissues containing kreatin and xanthin, proliferation of leucocytes occurs immediately; and the greater the injury, the greater the cell-proliferation.

We think that if the problem is carefully con-

sidered, and, better still, if these mitotic divisions are actually seen as they occur in response to chemical agents, it will be appreciated that there is a strong probability that cells only divide when they are made to do so by an exciter of reproduction.

The active auxetics are contained in "the remains of dead tissues." Globin is in reality "the remains of a dead tissue," for it is obtained by the decomposition of hæmoglobin, and hæmoglobin is contained normally in living red cells. Doubtless the constituents of the molecules of kreatin, xanthin, and the active principle of globin are present in living protoplasm; but they may not be present, presumably, in the same combination or form as they exist in kreatin and xanthin. Possibly it is only after death that these substances are produced, in which case it would follow that a cell will not reproduce itself by virtue of the constituents of its own living protoplasm; but it is necessary for it to absorb fresh active agents from the dead remains of its neighbours.

Many points are now explained. When it is required that an indolent healing surface shall heal well, we scarify it, as exemplified in the operation of Thiersch grafting. If a fractured bone will not unite, the ends are rubbed together or actually "freshened" by operation, to produce callus; and callus is really a tissue made by the proliferation of cells. When we scarify or freshen a surface, we merely cause destruction, and thereby set free exciters of reproduction. If a part of the body is bruised, hæmorrhage occurs; and, as is shown by the pigmentation, the hæmoglobin set free

from destruction of red cells which have been shed into the injured tissues is decomposed, and globin is thus locally produced. The cell-proliferation of healing must then occur in response to it, and the remains of other tissues which have been killed in the injury.

The proliferation of cells, however, is not confined to the cell-proliferation of healing. It will be shown that epithelial cells will also respond to auxetics, and probably some if not all other cells also respond to the soluble remains of their neighbours by reproducing themselves. It is true that globin does not exist in the cornea, for here there is no blood supply, and consequently no hæmoglobin until some time after the injury. Still, if the cornea is injured the corneal cells must be injured, and the cell-proliferation of healing occurs in response to the remains of the injured cells.

Irritation is always followed by cell-proliferation. Irritation means damage, and damage means cell-death. Cell-death sets free kreatin, xanthin, and other auxetics, and the cell-proliferation is caused by their absorption by the neighbouring living cells. The greater the damage, the greater will the cell-proliferation be.

Cell-division is apparently an automatic phenomenon—not in the sense that it is due to some intrinsic function or duty of a cell's protoplasm, but automatic in that the death of one cell will cause the reproduction of its living neighbours. If we may speak of the act of cell-division by mitosis as the "birth" of cells, then we may say that the number of births of cells in the body depends on the number of deaths. The greater the number of deaths, the greater



the number of births. If an individual cell dies, its death causes its neighbours to multiply to supply the deficiency; but if the cell-death is extensive owing to damage, the proliferation of those cells which have not been killed will also be extensive, and this proliferation will now be extended to that of the white blood-corpuscles which have been shed during and after the injury; and the result will be the cell-proliferation of healing.

Judging from the experiments which have been made, it may also be assumed that since the number of cell-births depends upon the number of cell-deaths, and since an increase in the number of births must increase the number of deaths, it follows that the number of deaths must also depend to some extent on the number of births. Presumably, if once cell-division is set going in a tissue or in a part of a tissue, that cell-division will go on increasing until something restrains it. Elimination from a tissue of tissue fluids would restrain it; for if the soluble remains of dead tissues become quickly eliminated, the diffusion of the constituents of these fluids into the cells would also be arrested, for that diffusion varies directly with the factor *time*. In a damaged tissue the vessels and lymphatics are also damaged, and elimination may be impaired; hence the remarkable cell-proliferation which leads to "granulation tissue." In an injury of any part except the cornea, coagulation of the shed blood occurs; the red cells become laked, and ultimately the hæmoglobin is evidently decomposed, as evinced by the pigmentation which will always be seen even in a

bruise. The globin so produced will assist in promoting the cell-proliferation of healing.

Such is the explanation of the cause of cell-division in the human body as demonstrated by *in-vitro* experimentation. But we think that we may go farther, and suggest that the initial multiplication of the cells in the human embryo may also be caused by a chemical auxetic. Spermatazoa contain extractives. Possibly it is these extractives, set free from this spermatozoa, which, after fertilisation, give rise to the subsequent cell-division in the ovum from which the embryo is built up. Once the cell-division has started, it will go on in response to the cell-deaths which sooner or later must occur.

As we have pointed out, kreatin is not by any means the only auxetic contained in the remains of dead tissues, and it is yet to be proved that there is not some specificity in cell-reproduction due to some at present unknown substance. We know from the study of heredity that certain characteristics are carried in the ovum and in the spermatozoon, and if they are so carried, doubtless other chemical auxetics, far more complex than kreatin, may be carried too.

In the meantime we think that the knowledge that dead tissues cause cell-proliferation is sufficient to give an inkling as to the cause of benign growths. A sudden cell-death occurring in a tissue will cause proliferation of neighbouring cells. Of course, if the initial cell-death is extensive, the cell-proliferation of healing will occur which ultimately leads to the production of connective tissue, which in itself may

prevent undue extension of the proliferation of the normal tissue-cells. But supposing for some reason, such as a slight injury, a local cell-death takes place: it would cause increased proliferation of local cells, and so form the basis of a tumour. Once this growth is started, it will go on until, by causing "irritation" or, to be more accurate, extensive cell-death, it may now induce the cell-proliferation of healing round it, and so, by the formation of connective tissue, cause its progress to be arrested by a capsule. A benign tumour is probably due merely to some localised cell-death in the first place, and it is remarkable how frequently there is a history of injury in these cases. But there is also no doubt that the onset of benign growths, and other cell-proliferation too for that matter, must be controlled to some extent by nervous influence. Possibly this nervous influence may be actuated by the nervous control over local elimination. Quite recently a paper appeared in *The Lancet* on a case of bilateral benign tumours;<sup>1</sup> and this can only be due to some central control over the local causes of cell-division.

Fibroids of the uterus occur only during the years of menstrual activity. During this time the uterus periodically becomes enlarged, followed by reduction in size. This reduction and quiescence must be accompanied by death of living cells, and presumably it is this death which, if elimination of the products of katabolism is impaired, may lead to excessive pro-

<sup>1</sup> See a paper on Bilateral Tumours by W. Roger Williams in *The Lancet*, Feb. 12, 1910.

liferation of the remaining living cells, and so cause the growths known as fibroids.

The foregoing conclusions and deductions have been arrived at from experimentation *in vitro* with individual cells. As pointed out in a former chapter, conclusions derived from *in-vitro* experimentation are not in themselves sufficient to prove a point. Because we can induce cell-division in individual cells on the microscope stage with certain chemical agents does not prove that the same division will necessarily occur *in vivo* in the same cells in response to the same agents. But, fortunately, *in-vivo* experimentation with these agents has not been impossible, and the proof that these agents, or rather some of them, do actually cause proliferation in the body is now at our disposal. In the wards of the Royal Southern Hospital at Liverpool cases of chronic callous ulcers of the legs were admitted, and have been treated in the first instance with saturated solutions of globin. The globin was applied to portions of the ulcers by dipping pieces of sterile gauze in the solution and applying it direct to the ulcerated surfaces. Granulations immediately appeared in response. In the short space of three or four hours a difference appeared between the extent of the granulations in the treated as compared with the untreated portions of the sores. In twenty hours the difference was marked. Granulomata have been produced in a day or two by means of globin.

Others suggested that the proliferation was not necessarily due to the globin, but to the "irritation"

of the gauze, in spite of the fact that ulcers have been treated with gauze all over, but only a part of them with globin added, and the proliferation occurred to the marked extent only where the globin was. We therefore discarded gauze or dressings altogether, and repeated the experiments. In a case where there were several ulcers on one leg the surfaces of them all were scarified, and small pieces of dried globin were "dotted" all over one ulcer. The cell-proliferation occurred to a marked extent in that ulcer, but only to a much less extent in the others which were not so treated.

Globin thus applied to a healing surface causes a scab to form very rapidly (figs. 111, 112), and the cell-proliferation goes on beneath it. This scab forms in an hour or two, whereas, if no globin is applied, it takes several days for a scab to form on an ulcer which has no dressing on it. Globin also causes extensive proliferation of the epithelium from the sides of the ulcer.

Unfortunately suppuration occurs under the scab, no matter how "clean" the ulcer may be when the globin is applied. The onset of suppuration, however, has been delayed by preparing the globin with aseptic precautions throughout, thus: A solution of hæmoglobin is decomposed by boiling, and filtered, and the globin solution is concentrated until it precipitates by further boiling. It is evaporated to dryness at a temperature of 60° C. and immediately sealed into sterile glass tubes. Even with these precautions, suppuration usually occurs under the scab in the course of a few days. The scab is then removed with fomentations,





FIG. 111.—To show the way in which globin is “dotted” over the surface of an ulcer.



FIG. 112.—To show the scab formed by the application of globin to an ulcer.





and when the sore is clean it is once more scarified, and fresh sterile globin is again "dotted" over its surface. This procedure can be repeated until the ulcer heals. During the scarification it is better to draw blood. Latterly this treatment of ulcers has been improved by using powdered globin (five parts), mixed with two parts (by weight) of kreatin, a mixture which produces more marked proliferation than pure globin.

Many ulcers have now been treated by this method, and we think that we can say safely that it causes more rapid healing of them than if they were treated in the usual way. Callous ulcers will usually heal by themselves if the limbs are kept at rest, and it was suggested to us that the cell-proliferation produced by globin was in reality due to the fact that the patients were kept in bed. This suggestion was disproved, however, by the production of extensive proliferation in one part of an ulcer by means of globin in a patient who was made to walk about during the treatment. Lastly, granulations have been induced by extracts of suprarenal gland.

It should be mentioned that globin, kreatin, etc., when applied to a healing surface will not only cause proliferation during the application; but once the multiplication has started, it will continue "automatically," even though the application of the auxetic is discontinued. This point has frequently been seen during the experimentation with ulcerated legs, and it is proof that the proliferation of cells is "automatic." There can be no doubt that once proliferation is started

in an ulcer, an increased number of deaths is occasioned, which in its turn still further increases the proliferation, as seen in the ulcers once treated with globin.

The application of dry globin to a scarified sore has elicited the interesting fact that it will convert the dark venous blood drawn by the scarification into the bright and red arterial variety, and the scars resulting from the treatment appear to be exceptionally firm and unlikely to break down again.

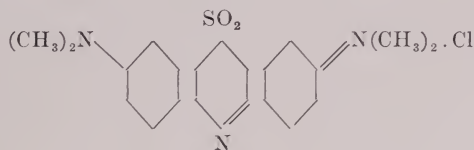
This form of treatment, however, must be carried out with care, and suppuration not allowed to continue for long in the presence of an auxetic, for, as will be shown in the next chapter, there is a possibility of malignant proliferation occurring in place of the normal one if the products of decomposition become pent up in the neighbourhood of proliferating epithelial cells.

These experiments afford conclusive proof that the cell-proliferation of healing can be caused by the chemical auxetics, kreatin and globin, and that the deductions made from the prolonged experimentations with the *in-vitro* method described in this book are correct. The possibility of the mitotic divisions induced on the microscope slide being in the nature of "freaks" or being due to death-struggles is disproved. As a matter of fact, these possibilities practically fell to the ground when mitoses were induced by extracts of dead tissues. One could conceive that a purely artificial substance like azur dye might cause mitosis by exciting the cells greatly just before death; but we think that in all probability the aniline dye contains some constituent

which possibly resembles the molecules of the natural auxetics.<sup>1</sup>

The fact that the cell-proliferation of healing is caused by chemical agents contained in the soluble remains of dead tissues will, we confidently believe, be the means of solution of many problems which at present confront the investigator in pathology and perhaps in physiology also. It is a fact about which there can be no doubt whatever.

<sup>1</sup> The formula of azur dye (*Cent. f. Bakteriologie*, Bd. xxix., 1901) is:



## CHAPTER XVI

THE AUGMENTED DIVISIONS INDUCED BY PUTREFACTION OF THE EXTRACTS ARE DUE TO THE ALKALOIDS OF PUTREFACTION—A THEORY THAT CARCINOMA AND LYMPHADENOMA MAY BE CAUSED BY THE MIXTURE OF THE AUXETICS OF CELL-PROLIFERATION WITH CHOLINE OR CADAVERINE — AN EXPLANATION OF THE AGE-INCIDENCE, METASTASES, AND OTHER FACTS KNOWN CONCERNING CANCER—THE NECESSITY FOR A CRUCIAL EXPERIMENT TO PROVE THE THEORY

IN Chapter IX. it was pointed out that there is an intimate association between “chronic irritation” and the onset of cancer. As just shown, “irritation” means cell-death, and cell-death is followed by cell-proliferation. When a tissue is the seat of chronic irritation, the cell-proliferation of healing must be going on in the damaged site owing to the presence of the remains of the dead cells. The proliferation occasioned by irritation is in reality due to the auxetics, some of which are kreatin, xanthin, and that contained in globin, which are set free by the death of some of the cells. This will explain why an ill-fitting boot will give rise to

a "corn," and to the "induration" of a tissue which is under pressure or being chronically irritated. In reality "irritation" must be followed by chronic cell-proliferation due to the auxetics produced.

Now, the chief characteristic of cancer is that it consists of a growth of cells which are proliferating excessively. Every cancer is a growth which infiltrates the surrounding tissues; and this growth occurs probably in every instance in a site in which there is chronic irritation—or rather where there is chronic cell-proliferation of healing due to auxetics.

One may suggest, therefore, that since the proliferation of chronic irritation is due to the auxetics produced by cell-death, the proliferation of cancer is also associated with them. The proliferation of chronic irritation, however, is a normal one, whereas that of cancer is a malignant one. If the cause of the normal proliferation is removed, then ultimately proliferation ceases; but if the irritation which predisposed to cancer is removed, the malignant cells appear to continue to multiply until the patient dies. Yet cancer-cells are cells of the body. They are not foreign parasites, and hence it may be that in a cancerous growth there is some other factor in addition to the normal ones. Therefore it may also be suggested that the onset of cancer in a normal healing site may be brought about by the presence of another agent in addition to the normal auxetics produced by cell-death.

Now let us return to the "augmenting" of the action of auxetics in promoting cell-division by putrefaction and by the alkaloid atropine. It is well known



that certain putrefactive bacteria in decomposing dead organic structures produce ptomaines and leucomaines. These substances are in the nature of alkaloids. The following are common ones:

Choline . . . . .	$C_5H_{15}NO_2$ .
Cadaverine . . . . .	$C_5H_{14}N_2$ .
Neurine . . . . .	$C_5H_{13}NO$ .
Putrescine . . . . .	$C_4H_{12}N$ .

Choline will, like other alkaloids, excite amœboid movements in leucocytes and lymphocytes, and so will cadaverine. In fact choline is just as effective as atropine in this respect. The best strength of choline to employ to excite amœboid movements in leucocytes and lymphocytes is one in which 10 cc. of jelly contains 0.01 gramme of the alkaloid in addition to the 10 units of alkali. Choline, however, is not very poisonous to leucocytes, and even 0.04 gramme will not kill them. Cadaverine also excites leucocytes, and 10 cc. of a jelly containing 1 cc. of a 1-per-cent solution of it is suitable for this purpose if 10 units of alkali are also present, the jelly-film being examined, of course, at the room temperature.

It may be remembered that it was through the accidental putrefaction of the extract of suprarenal gland that we were enabled to induce divisions with it by itself for the first time, and we now know that the reason for this was that the putrefaction produced the alkaloids choline and cadaverine in the solution of the extract, and that they, like atropine, greatly augment the action of auxetics in inducing cell-division.

In order to prove this point we now, in the first instance, used these pure alkaloids, choline and cadaverine, added to the extracts, and afterwards we combined them with kreatin and xanthin to induce augmented divisions.

If a jelly contains 0.01 gramme of choline and 10 units of alkali solution, divisions in lymphocytes can be induced if only 0.02 or even 0.01 gramme of kreatin is present (fig. 113). In fact, this alkaloid of putrefaction choline, like atropine, augments the action of auxetics about five-fold.

Using cadaverine in the strength given above, divisions in lymphocytes were induced if the jelly contained only 2 cc. of a 1-per-cent solution of kreatin.

It has already been mentioned that a mixture of atropine and an auxetic will give rise to asymmetrical mitosis in lymphocytes, and we have also found that these remarkable mitoses also are frequently induced by the augmenting action of choline and cadaverine (figs. 114-16). This point is of great importance, because it is well known that asymmetrical mitoses are frequently seen in cancerous growths.

So far the augmented divisions had only been induced in lymphocytes. It is true that there is a form of cancer which occurs in the lymphocyte class of cells of the lymphatic glands (lymphadenoma); and if it is a criterion that because a lymphocyte divides by an augmented asymmetrical division it is necessarily malignant,<sup>1</sup> then the combination in certain proportion between the causes of the proliferation of healing plus an alkaloid of putrefaction like choline must be a cause

<sup>1</sup> It has not been proved to be a criterion.

of lymphadenoma. In connection with this it is interesting to note that many years ago Trousseau<sup>1</sup> stated in his book that lymphadenoma often follows on a suppuration focus, and this view is upheld by many to this day. At the same time it must be remembered that no alkaloid has yet been made to induce a division by itself; it is essential for an auxetic to be present also. Alkaloids appear to be augmenters only of cell-division.

But our object was, if possible, to find the cause of carcinoma, and we therefore tried to see if our chemical agents would induce divisions in epithelial cells. Considerable difficulty was met in investigating this point. Epithelial cells will not live long *in vitro*; in fact, they usually die in a few moments, as far as can be seen. But at last we did succeed in inducing an early mitotic figure in two epithelial cells (as shown in the photographs, figs. 117, 118) from the vaginal secretion. We did not succeed in inducing the divisions with an entirely "natural" agent, for epithelial cells evidently require more auxetics than even leucocytes. The figure induced was seen when the epithelial cells were placed on a powerful jelly which contained azur stain, putrid extract of suprarenal gland, and atropine. *In vivo*, also, epithelial cells undoubtedly proliferate in response to globin and kreatin.

The fact was, therefore, proved that epithelial cells respond to the chemical exciters of reproduction, and it is possible that they may be subject to the same conditions as lymphocytes, and only respond to them.

<sup>1</sup> Trousseau's *Clinical Medicine* (Sydenham Society), 1872, vol. 5, p. 207.

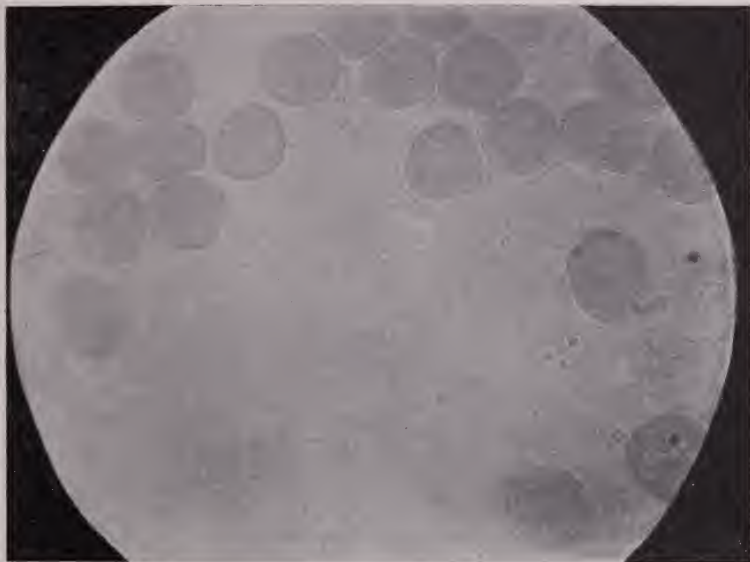


FIG. 113.—Mitosis induced by a mixture of kreatin and choline. No stain, extract, or atropine.

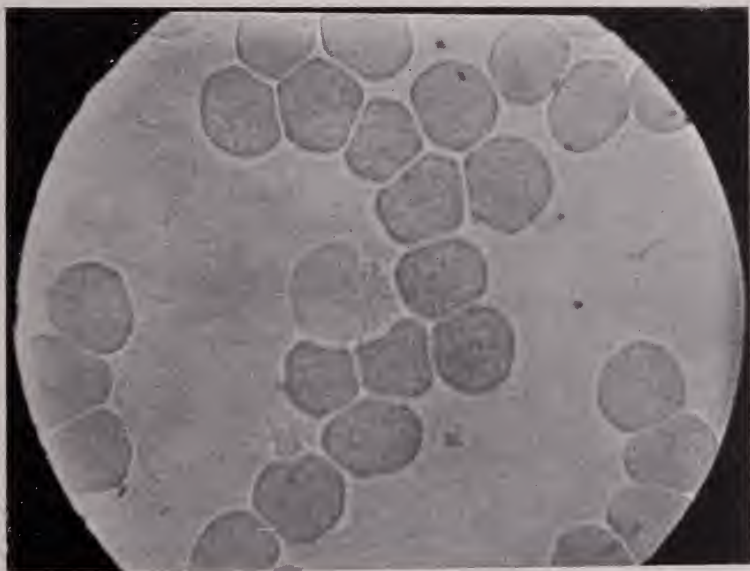


FIG. 114.—Asymmetrical mitosis induced in a lymphocyte by a mixture of suprarenal extract and globin, augmented by choline. No stain or atropine.



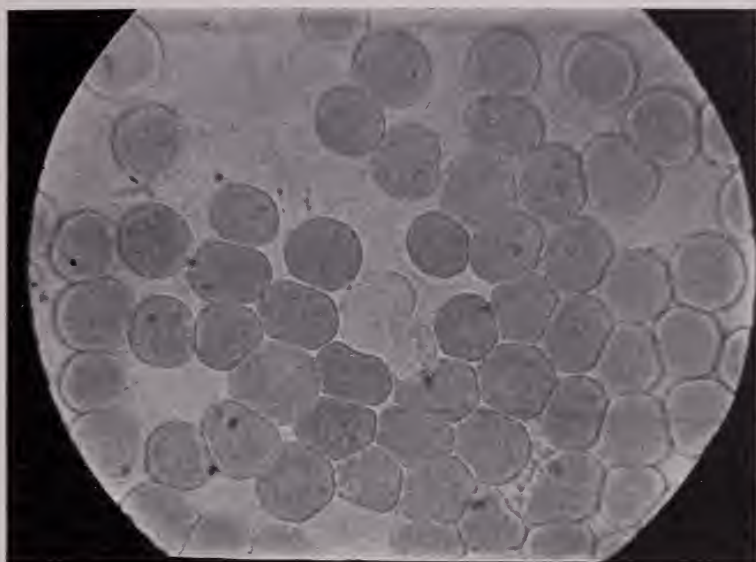


FIG. 115.—Mitosis in a lymphocyte induced by globin and choline. No stain or other auxetic.

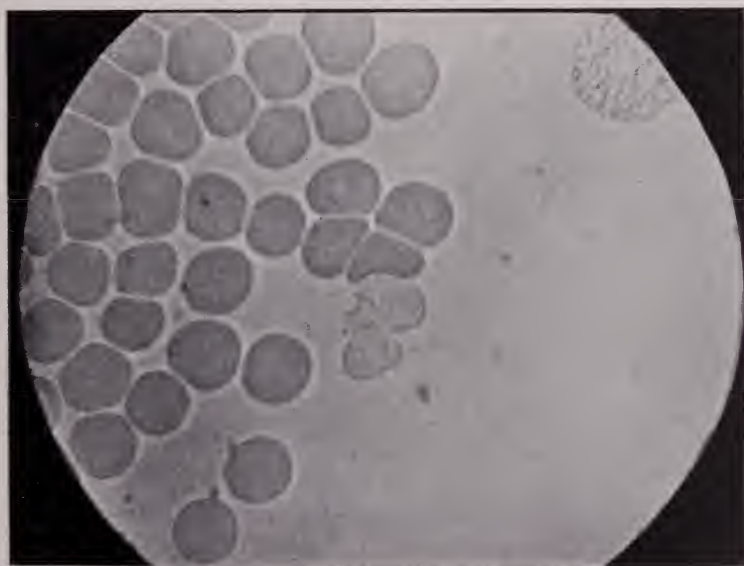


FIG. 116.—Mitosis induced in a lymphocyte by suprarenal extract and choline. No stain or other auxetic.







FIG. 117.—Mitosis induced in an epithelial cell by a mixture of stain and extract.

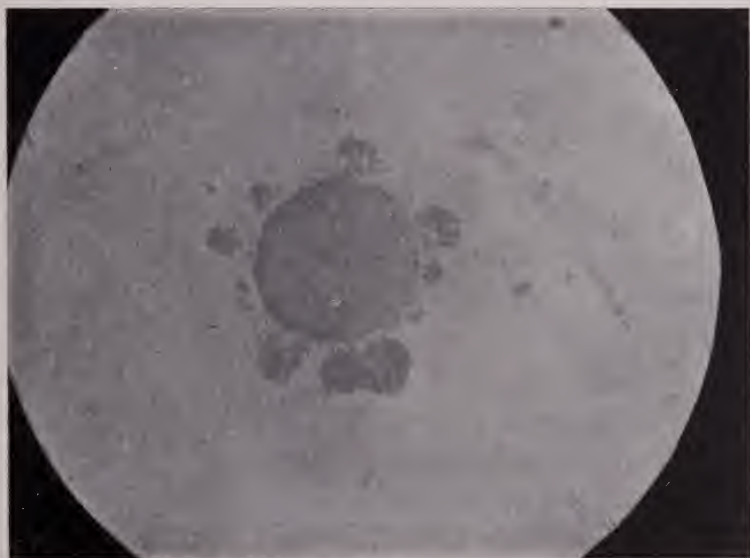


FIG. 118.—Early mitosis in an epithelial cell from the vagina induced by stain and extract.



The action of these auxetics upon lymphocytes is greatly augmented by the alkaloids choline and cadaverine, which are produced in a solution of an extract of a dead tissue as it decomposes. We now made some experiments to see if putrefaction also augmented the auxetic action of globin, and a solution of it was therefore allowed to decompose at the room temperature for about three weeks. A solution of globin of no matter what strength will not (as already noted) induce divisions by itself in ten minutes; it is necessary to add atropine. But when a 1-per-cent solution of globin had decomposed, it was found that now it would induce divisions in lymphocytes by itself in the experimental ten minutes. Whether this augmentation of the action of globin by decomposition is due to the production of choline and cadaverine or not, we are uncertain; for although we have tested<sup>1</sup> the decomposed solution for the presence of alkaloid, only a negative result has been obtained. This matter will require further investigation, for it is possible that other substances besides the alkaloids of putrefaction may augment the action of exciters of reproduction. We have so far obtained the best divisions by making up the 10 cc. of jelly with 0.5 cc. of a 2-per-cent solution of globin which had been kept open to the air of the room for three weeks.

Decomposition of organic solutions which contain exciters of reproduction will augment the action of the latter agents up to as much as five-fold; and in this case the divisions induced in lymphocytes are fre-

<sup>1</sup>The iodine and the mercuric chloride tests were employed.

quently of the asymmetrical variety. Cancer is a growth of cells which supervenes on an old irritated site where the cell-proliferation of healing has been going on for some time. Since cancer is an exuberant growth of epithelial cells—which will respond to auxetics—it is obvious that the quantity of the action of the normal auxetics present must be augmented in some way so as to give rise to the exuberant proliferation of malignancy. And lastly, the mitoses in a cancerous growth are frequently of the asymmetrical type. The combination of a normal auxetic plus an alkaloid of putrefaction and decomposition will cause not only augmented divisions, but it is important to note that these divisions tend to be asymmetrical in character.

Having arrived at this stage of our researches, these new facts were carefully considered to see how they harmonised with the well-known features which are associated with cancer, and we shall now discuss them.

The fact that cell-division is caused by substances contained in the remains of dead tissue throws light on the age-incidence of the disease.

In a paper published by us in *The British Medical Journal* on October 23, 1909, when we were aware of the fact that cell-division could be induced by an aniline dye, and that its action could be augmented (which was all we knew then) by the remains of a dead tissue, we appreciated that the remains of dead tissues might be a predisposing factor in the cause of carcinoma. We may as well quote the passage from that paper, for it shows the possible relationship between the remains of

dead tissues (the products of katabolism) and the age-incidence of cancer.

The body is mainly composed of living cells, and they constitute an elaborate combination of living factors. We know that in certain tissues these cells are continually dying and being replaced, so that it is evident that birth and death must be going on incessantly in the body. What happens to the dead cells? They of course liquefy and become disorganised, and their constituents are presumably excreted or converted into other compounds. While this is happening it seems probable that some of the products of the remains of dead cells may be absorbed by their neighbours, for it must be remembered that the diffusion of substances into living cells appears to be a physical process over which they exercise no control. There are doubtless some cells which remain alive for long periods; for instance, it has been estimated (and we are informed that it is practically certain) that some cells of the central nervous system live throughout the life of a man. Many cells, however, only live a very short time, the length of their lives perhaps varying in different parts of the body, so that the remains of dead cells are probably always present in the body fluids. In this connection, however, we have to keep in mind the physiological curve expressive of the relationship between anabolism and katabolism. There are only three stages of life if it is viewed from this point of view, the first terminating at about the thirtieth year, when a man reaches his prime, and up to which period cellular birth must preponderate over its death-rate. For some years it may be suggested that anabolism and katabolism remain balanced;



and that after the age of 40, quite physiologically, so that nothing occurs to make a man aware of it physically, these conditions begin to be reversed and more of the products of katabolism—that is, the remains of the dead cells—tend to exist in the body-fluids than was the case before middle age.

Here we have a fact incidental to the cancer period which suggests the possibility that these products of katabolism may in some way predispose to the onset of malignancy. It cannot possibly be suggested that they are the cause of the disease, for if such were the case everybody over the age of 40 would die of cancer; but assuming that some product of katabolism may possibly favor the onset of the disease, we may enlarge upon the speculation and say that it is a certain morphological (or chemical) element in a dead cell which may be the agent. For the sake of argument it may be derived from either the cytoplasm, the cell-wall, the nuclear wall, or the linin, or it may be the chromatin itself.

It is now known, of course, that the products of katabolism actually contain causes of cell-reproduction; and it follows that if it is correct that these products are in excess after the age of 40, there must be, *ceteris paribus*, a greater inclination to cell-proliferation in a tissue after that age than before it. To produce cell-division it is necessary for a cell to absorb a certain quantity of an auxetic, and to produce augmented asymmetrical divisions by an alkaloid it requires a certain combination (as already specified) between the alkaloid and the auxetic. If cancer is due to this combination, it is possible that before the age of 40 there is not usually sufficient free auxetic to produce

the right combination, for we have shown that alkaloids by themselves are not effectual in inducing cell-division. On the other hand, after the age of 40 the slight physiological increase in the quantity of auxetics present in a tissue, owing to excess of products of katabolism, may just supply the required quantity of auxetic to produce the right combination between them and an alkaloid, should the latter be present. It has already been suggested that the onset of cancer may be partly due to the oversetting of a normal balance.

In connection with this point we would recall the fact that cancer seems to attack persons who are prematurely aged, especially those who are subject to such diseases as the atrophic form of osteoarthritis—a fact which seems to bear out this explanation of the age-incidence of cancer.

Conversely, it has been shown that cells with a lowered vitality require more of an auxetic to produce cell-division in a given time than normal cells; and this may explain why cancer does not so commonly occur in the aged and infirm, for although the right combination of the cause of the disease may be present, it is not present in sufficient strength to produce malignant proliferation in cells which have lost their vitality to some extent.

The suggestion that cancer may be due to putrefactive decomposition of the remains of dead tissues in a chronic healing site will harmonise with the fact that cancer occurs commonly in certain sites. Carcinoma occurs most frequently in the breast, uterus,

mouth, stomach, intestinal tract, and rectum. A chronic healing focus in the rectum, mouth, or intestine may readily be associated with decomposition products. As already pointed out, chronic irritation means chronic cell-proliferation of healing due to the auxetics contained in dead cells, and in the rectum, intestine, and mouth further decomposition with the gradual production of alkaloid must easily occur in a chronically injured site in these regions. It is interesting to note that cancer of the pancreas nearly always attacks the "head" of that gland, namely, that part of it which has nearest access to the intestine. In the rectum "irritation" must be of frequent occurrence by the impaction of fæces, and this in itself will obviously render this part of the alimentary canal a common site for malignant disease—and it is one of the commonest places for it. In the mouth decomposition readily occurs, and how commonly one sees carcinoma of the tongue, lips, fauces, etc.

Syphilis is undoubtedly a predisposing factor. Syphilitic lesions of the mouth, which, of course, are accompanied by healing, are of very common occurrence, and it is possible that choline is produced in tertiary syphilides. If choline is produced by the action of *Trypanema pallida*, then the cause of syphilis may be a predisposing cause of cancer—that is, if the argument is correct that the alkaloid choline in certain combination with auxetics gives rise to malignant proliferation.

The breast and *cervix uteri* are localities which are very prone to cancer, and in these organs destruction

of tissues occurs to some extent every month until the climacteric, when great involution takes place. It is during this latter period that the onset of carcinoma is favoured. It is a remarkable thing that cancer almost only occurs in these parts in parous women, whereas in nulliparous women they are comparatively free. We do not think we are going too far in suggesting that in parous women, when the ducts of the glands of the breast and uterus have been—so to speak—opened up, access is now afforded to the organisms of decomposition and putrefaction. In nulliparous women, when these organs have remained functionless, the ducts of their glands are more likely to be closed to invasion from without.

In any site, however, the products of katabolism may determine the age-incidence of carcinoma.

One cannot assert that the alkaloid choline, or cadaverine either, are only produced in a damaged site by the action of putrefactive organisms.. It was owing to decomposition by putrefaction of extracts of dead tissues and globin that we were enabled to obtain augmented asymmetrical cell-divisions with these alkaloids, but it is possible that these alkaloids—or others equally effective—may be produced by other agencies; and if so, provided the contention is correct that the alkaloids help to cause carcinoma, other agencies besides putrefactive organisms may cause the disease. The point is an important one in view of the controversy as to whether cancer is a “parasitic” disease. In any case these alkaloids can be produced by more than one class of organisms, and we have

pointed out that the cause of syphilis may also produce one of them—in fact, General Paralysis of the Insane has been said to be due to choline. Hence, if our contention is correct, cancer can hardly be said to be due to a specific parasite.

The mere fact that the alkaloids were being produced in a chronic healing site would not necessarily cause in it augmented proliferation. The alkaloids and the auxetics will have to be present in certain proportion; and since the production of this proliferation necessitates the diffusion of the combination into the cells, time is an essential factor. In all probability it would be necessary for the decomposed remains of dead tissues to be pent up to some extent for a considerable period.

The suggestion that cancer may be due to the combination of auxetics and ptomaines will offer an explanation of the cause of death from the disease. If the malignant cells and healing site are completely removed, the patient may recover; but if this is not the case, recurrence will, of course, take place, for in removing the growth a fresh healing site is produced, and the original decomposition may go on in it. Putrefaction of the remains of dead tissues may occur in a healing site without visible suppuration. The *Bacillus subtilis* does not produce pus, yet it will produce choline. It may be these ptomaines which ultimately cause the death of the patients by poisoning them; for if decomposition sets in in a damaged site, unless steps are taken to remove it, doubtless the decomposition will usually go on.



The possibility of carcinoma being due to the combination of alkaloids and auxetics will also explain the reason for the way in which malignant growths frequently "break down." As shown by *in-vitro* experimentation, cells can only withstand a certain quantity of the combination. If excess is forced into them, they will die. Even globin itself is very poisonous to leucocytes and lymphocytes if it is in excess. If this excess was present in the body, it would cause cell-death unless the excess was removed, and the cell-death would only aggravate the trouble, especially if the lymphatics were blocked by malignant cells. Ultimately, of course, the growth would "point" and break down. A certain amount of local cell-death will cause increased cell-proliferation; but after a certain stage is reached, breaking down must occur with subsequent ulceration.

Up to a certain point, therefore, the greater the malignant proliferation, the more cell-death will there be, and the more will the disease be aggravated. And the aggravation may be increased by the chromosome granules which cells appear to discard when they are excessively prolific. These chromatin granules may contain kreatin, and they will therefore merely supply more auxetic for the neighbouring cells.

The phenomenon of metastasis in cancer is an important factor to be considered in conjunction with the other facts. The invasion of lymphatics by cancer may be due to the combination of auxetics and alkaloid being passed through them from the original healing



site. We have seen amœboid movements in cancer-cells in response to alkaloids, and possibly this may assist in the infiltration of vessels and tissues and so predispose to metastasis. A striking fact known about secondary growths is that in the arrangement of the cells they resemble the primary ones. We think that this can be explained only by embolism. If a secondary growth in another organ was a fresh cancer, it is difficult to imagine how it could possibly resemble the primary ones in the arrangement of the cells. Metastases practically only occur in the later stages of carcinoma when the lymphatics have been extensively invaded. In benign growths one rarely if ever sees the vessels invaded by cells, and presumably this is the reason why secondary tumours do not follow. The extensive researches which have been done by others in transplanting tumours in mice have thrown considerable light on the nature of secondary growths. In transplanting a tumour from one animal to another, it seems to us that one is in reality producing a secondary tumour. Now, to effect this, as is well known, it is necessary that the cells of the tumour should be alive; the transplanting of dead cells will not cause a secondary growth. This knowledge harmonises with our suggestions as to the cause of cancer. If one inoculates an animal with dead cells, although the organisms of putrefaction may be present among them, the remains of the dead cells are soon removed from the inoculation site and the production of augmented auxetic must cease. Normal healing will take place before the putrefactive organisms have had time to restart and pro-

duce choline, cadaverine, etc., for it is known<sup>1</sup> that to produce these alkaloids it takes at least a fortnight. If, on the other hand, a portion of a *living* primary growth is transplanted, the living cells will continue to multiply in response to the auxetics produced by the cell-death which continues to occur among the malignant cells which have been inoculated. In transplanting a malignant growth, one must transplant some putrefactive organisms along with the malignant cells, and in the spaces between the cells the combination of auxetics and alkaloids must be present from the outset and be continuously produced without interruption, because a living growth is transplanted. For a secondary growth (or a metastatic one) to occur, it is necessary for living cells to be transplanted; and we believe that it is also necessary for organisms to be transplanted within it, so that the causes of the augmented proliferation continue to be supplied without interruption.

There is another possible explanation of a metastatic growth which should be mentioned. It has been suggested by others, who, of course, were unaware that cell-division in the body is caused by chemical agents, that once a cell becomes a malignant one, its daughter cells will also be malignant. This would mean that a cell, in acquiring malignant characteristics, would transmit those characteristics to its progeny. This would be a "mutation"—an acquired characteristic suddenly becoming hereditary for all succeeding generations; an event which we think is most unlikely to

<sup>1</sup>It must be remembered that these organisms may have nothing to do with either sepsis or suppuration.

occur. It is difficult to imagine how a cell, having started augmented divisions in response to a combination of alkaloid and auxetics, could in its subsequent generations continue to divide by augmented divisions when the cause of the augmentation is absent. We have shown experimentally that if the supply of auxetic to a cell ceases, the cell-division also ceases. This experiment tends to dispose of the expressions "first (heterotype) divisions and subsequent (homotype) divisions," which in reality imply a mutation. We think, therefore, that a metastatic growth consists of a portion of the primary one transplanted elsewhere along with some of the original cause of its augmented proliferation.

It is possible that in the later stages of cancer the body-fluids may contain considerable amounts of alkaloid, derived from the primary growths, which might, in the event of a fresh healing focus occurring anywhere, be sufficient to act in combination with the new local auxetics, and so cause another "primary" growth. If such occurred, it would probably be mistaken for and called a secondary growth.

Lastly it may be mentioned that if cancer is due to putrefaction occurring in a chronic healing site, there may be something in the view upheld by many, that the disease occurs frequently in certain localities or even in certain houses. Doubtless putrefaction will occur more readily in certain places, because the bacteria of putrefaction may infest the air there. In connection with this I may recall the remark—already noted—which was made to me by Sir William

MacGregor, that he had never seen a case of cancer among the Esquimo.

The "error of random sampling," however, must be considered with the question of the "local incidence" of cancer. Very large figures would have to be studied before one could say conclusively whether the incidence of the disease is actually greater in some localities than in others, and experimentation with animals in the confines of the laboratory cannot, we think, determine whether putrefaction is more likely to occur in one place than in another. Still, the remark of Sir William MacGregor is striking, because it is clear that putrefactive bacteria cannot be present to so great an extent in the Arctic regions as in temperate and tropical climates.

The above consideration led us to believe that our researches did harmonise with the facts known about carcinoma. The fact that cell-proliferation is caused by auxetics contained in the soluble remains of dead tissues offers for the first time an explanation of the remarkable age-incidence of the disease; and the augmented asymmetrical division induced by these auxetics combined with alkaloids of putrefaction seemed to be a reasonable explanation of the cause of cancer. Proof was wanting, however. Cancer-cells have been seen frequently to divide by asymmetrical divisions, but because one can induce these mitoses in cells is not proof that one is necessarily inducing malignant proliferation.<sup>1</sup>

<sup>1</sup> As a matter of fact, the five-fold augmentation by alkaloids is a more important consideration than the asymmetrical mitoses induced by them.

Deductions from experimentation *in vitro*, no matter how well they may harmonise with known facts, are not sufficient to act as a basis on which to proceed to find the prevention and cure for the disease. It is necessary at least to try to prove one's work definitely. To accomplish this would not be, we knew, an easy matter. It would be necessary to produce a cancerous growth in healthy animals with the substances which were believed to be the cause of the disease. The chemical auxetics, in correct combination with an alkaloid of putrefaction such as choline, would have to be inoculated into or applied to an animal, and before one could say that the combination is a cause of cancer a malignant growth would have to appear at the site of inoculation. The experiment would have to be frequently repeated, and careful precautions would have to be taken against possible fallacy.

It was realised that it would be quite useless merely to inoculate a solution of, say, kreatin and choline subcutaneously into an experimental animal, because it is obvious that such a solution would rapidly be excreted, and we know from *in-vitro* experimentation that before a cell can divide, either by a normal or an asymmetrical division, it must be subjected to the chemical agent for a certain length of *time*. It would be necessary to create a sore, because a chronic healing site is essential; and this would not be readily accomplished in experimental animals, which are not easy to keep quiet, and in which the local application of substances to sores offers practical difficulties.



Moreover, the question whether the lower animals suffer from true cancer is still controversial. I therefore considered whether it would be possible to try this crucial experiment on a human being. If it were possible, and if it were successful, the point might be proved conclusively. At first sight the suggestion seems to be an outrageous one, but the experiments to be related in the next and last chapter, which had been carried out for several months past, revealed a method by which I considered that an attempt might be made to put this crucial experiment to the test.



## CHAPTER XVII

INHIBITORY ACTION OF BLOOD-SERUM ON AUXETICS—  
MEASUREMENT OF THIS ACTION—THE TREATMENT  
OF SOME CASES OF CANCER BY THE ADMINISTRATION  
OF DEFIBRINATED BLOOD—DESCRIPTION OF  
THE CASES—THE TREATMENT OF A MALIGNANT  
ULCER BY MEANS OF GLOBIN—AN ATTEMPT TO  
MAKE THE CRUCIAL EXPERIMENT—CONCLUSION

It is now (August, 1910) more than six months since it was ascertained that leucocytes and lymphocytes divide in response to the auxetics contained in the remains of dead tissues and in globin. When this fact was appreciated, the question arose as to why these cells, when they are removed from the peripheral circulation, had never been seen in the act of cell-division. White blood-corpuscles were discovered by Hewson in 1773; in 1846 Wharton Jones first described them as granular and nucleated cells (Buchanan). Since then they must have been seen by every student of medicine, but no one, until divisions were induced in them by us, had ever seen one of these cells divide.

Hence it is obvious that these cells do not divide in the peripheral circulation, for their mitosis occupies a certain amount of time; and if this mitosis occurred at all in the peripheral blood, it *must* have been seen during the century and a half in which these cells have been constantly examined by many thousands of workers. Now, the division of these cells is caused by the auxetics contained in the remains of dead tissues and in globin, and it also is certain that the peripheral blood must contain some free remains of dead tissues and globin. Hence white blood-corpuscles ought to be frequently seen in the act of division when they are removed from it. But they are not so seen. Had it been seen, the real nature of the Altmann's granules and the "lobes of the nuclei" would have been apparent many years ago.

We think that there can be only one explanation for this, which is that the action of the auxetics in the peripheral blood is restrained in some way. It appears to us to be reasonable to suppose that cell-proliferation in the peripheral circulation must be prevented in some way. If it were not, the approach of old age or a chronic suppurative focus with destruction of tissue might cause indiscriminate cell-proliferation in the vessels and capillaries, with disastrous results; for these vessels might ultimately become blocked. We therefore made some experiments to see if blood-serum does actually restrain cell-division.

In the first place, 2 cc. of sheep's serum was added to auxetic jelly composed of azur dye, atropine, and suprarenal extract. In order to prevent coagulation of

the serum in this and the subsequent experiments, the serum was added to the jelly after the latter was boiled and before it had cooled to such an extent as to prevent it setting on the slide. It was found that the serum did not prevent the cell-division induced by the azur stain.

The experiments were then repeated with a jelly which contained suprarenal extract, but no stain or atropine. The jelly was first tested, and mitotic figures induced in lymphocytes with it. The jelly contained 0.2 gramme of suprarenal extract, and it was found that if it also contained 0.5 cc. of serum the auxetic action of the extract was not stopped; but if it contained 2 cc. of serum the auxetic action of the suprarenal extract was completely inhibited.

The experiments were then repeated with an auxetic jelly composed of a mixture of 1 cc. of a 1-per-cent solution of kreatin and 1 cc. of a 1-per-cent solution of choline; and it also contained 10 units of alkali solution. With this jelly it required the addition of 2.5 cc. of sheep's serum to prevent it causing cell-division.

Using human serum, it required 2 cc. of it to stop the action of 0.2 gramme of suprarenal extract by itself. 1 cc. of serum will stop the action of the combination of 0.01 gramme of kreatin and 0.01 gramme of choline; 1 cc. of human serum will stop the action of 0.5 cc. of a 2-per-cent solution of globin which had been allowed to become putrid, and which would, by itself, induce division in lymphocytes.

Hence it is apparent that normal blood-serum

actually has the power of preventing the "natural" auxetics from inducing cell-division; but it has no inhibitory action against atropine or azur dye. The restraining power of serum can be measured as shown, and it is possible that this power varies with individuals, a point which remains to be determined.

It was also ascertained that the restraining body in serum does not combine permanently with the auxetic and so prevent its action. Jellies were prepared with suprarenal extract with kreatin and choline, which induced divisions in lymphocytes. The right amount of serum was added to them just before the jellies cooled, and it was noted that they stopped the auxetic action of the jellies. The same jellies were then boiled and the serum proteins precipitated. On making specimens again from these jellies, it was now found that their auxetic power was re-established. Hence it is obvious that the restraining body in serum is *not thermostable*.

Lastly, it was found out that 1 cc. of serum contained in 10 cc. of jelly which also contained 1 cc. of a 1-per-cent solution of choline stopped the kinetic action of the latter in exciting amoeboid movements in leucocytes. If the jelly was boiled, however, the action of the choline was restored.

These experiments are very constant in their results. Careful controls were made throughout. We think that by means of them the restraining power of different sera could be measured with a certain amount of accuracy. What the nature of the restraining body in serum is we have no opinion to offer. It should be

noted that some time ago Bashford and Murray showed that serum had the power of restraining the growth of secondary transplanted tumours in mice.

In addition to the restraining action of serum on the cause of cell-division, we also considered the work of Gaylord and Clowes of the New York State Cancer Research, Buffalo, and of Bashford and his assistants at the Imperial Cancer Research in London, who have shown experimentally that the transplantation of living growths in mice protect them to some extent against cancer. It was considered possible that this might be due to the fresh augmented auxetic produced by the transplanted growths' giving rise to an increase in the content of the restraining body. We therefore resolved to try to increase this body in cancer patients by deliberately injecting them with augmented auxetic combined with blood-serum. The way the combination was administered was by injecting 6 ounces of defibrinated sheep's blood per rectum every morning. The serum contains the restraining body, and it was argued that the red cells would be destroyed in the rectum, the hæmoglobin decomposed, and in time the globin would become augmented by the action of the bacteria present. It was presumed that the restraining body of the serum, the auxetic in the globin and in the remains of the white cells, and lastly, the products of the decomposition would be gradually absorbed, and that they might raise the content of restraining body in the patients; in other words they might act as a sort of vaccine.

We must admit that we were not very sanguine of



success when these experiments were first undertaken six months ago. They were undertaken more with a view to see what the effect of globin in this way was than with the object of obtaining a cure of the tumours from which the patients were suffering. But, as will be seen from the description of the treatment, the results have exceeded our anticipations. Unfortunately, since we did not expect any beneficial results, the cases were not the most suitable which could have been chosen, for both of them had "internal" growths which were inaccessible, and therefore we were at that time unable to prove conclusively that they were suffering from carcinoma.

The first patient<sup>1</sup> to whom the serum was administered was a woman (I. G.) aged 45 (admitted to the hospital on January 11, 1910), whose left breast had been removed in November, 1907, for a carcinomatous growth. She remained well until April, 1909, when she began to suffer from a severe pain in the region of the sacrum and left hip. She stated that this pain had since then become worse and that no treatment had relieved it. The left lower extremity from the hip to the ankle had for long been swollen and œdematous, and there had been swelling also in the abdomen. Any movement of the limb caused severe pain, and she had great difficulty in turning herself in bed. The patient was too ill to be weighed at the time of her admission, but she was manifestly wasted,

<sup>1</sup> These two cases were treated under the supervision of Dr. Macalister, who has kindly written these descriptions of them.



was anæmic, and had a worn expression. On examination there was manifest swelling on palpation in the left iliac region, and, examined per rectum under chloroform, a hard swelling could be felt attached to the anterior surface of the sacrum and sweeping round the wall of the pelvis towards the left side. An X-ray photograph confirmed the involvement of the sacrum. After treating her with mercurial inunctions and other remedies for a month without benefit, the defibrinated-blood injections were commenced on February 21, 1910. At first they were given in the evening, and were often followed by sickness and sometimes by actual vomiting; it was found that there was less disturbance when the injections were given in the mornings.<sup>1</sup> The sickness was so troublesome at first that the treatment had to be abandoned on March 3, and it was not until March 20 that it was again started and continued uninterruptedly. Gradually her pains improved, and the swelling in the leg diminished. (No opiates were needed after March 29.) On April 20 she could stand with very little pain, and she was weighed for the first time (109 lb.). Improvement continued week by week, she became bright and younger-looking, and on June 8 she weighed 115 lb. No pain could be elicited on pressure over the left iliac region, and the tumour seemed smaller. She maintained her weight, with some variation, for some weeks, and was able to walk about the ward without assistance until July 21,

<sup>1</sup> In these cases treated with rectal injections of defibrinated blood there has been sickness following the injection, but this has passed off as the treatment has been persevered with.

when she sprained her left shoulder and suffered severe pain in it. On July 19, by the patient's request, the treatment was discontinued, and an opportunity thus arose of observing whether the benefit which had resulted from it was maintained. There had been some sciatica-like pains in the leg since the beginning of the month, and during August these increased and the swelling and pain in the hip returned. Some tenderness and a tumour, apparently arising from the mediastinum and which grew rather rapidly, appeared in the mid-sternal region. By the first week in September, she had relapsed pretty much into the condition in which we found her at the time of her admission, but with the added pain due to the thoracic growth. The treatment has now been resumed.

The second case was that of a woman aged 54, who had suffered from indigestion for a considerable period, but severely for three months. There had been much vomiting, but never any blood. At the time of her admission (February 9, 1911) ingestion of food was immediately followed by severe pain, and often by sickness. She was very wasted, worn-looking, and anæmic. Weight 94 lb. On examining the abdomen a swelling could be seen and felt above the umbilicus. It was about the size of a tangerine orange. It was extremely tender, and moved with respiration. The stomach was very dilated, and presented peristaltic movements. There was pain on pressure over the pyloric region, but no tumour could be felt there. The stools contained altered blood (melœna). During the first fortnight after admission, when she had milk

and Benger's Food, the vomiting ceased, and she had less pain, but she lost four pounds in weight (90 lb.). The defibrinated blood was commenced on February 21, and until March 23 the weight fluctuated between 88 lb. and 91 lb., there being an occasional gain and then a corresponding loss; but on March 30 a steady advance commenced, the maximum weight being attained on May 8, when it reached 101 lb., *i.e.* a gain of eleven pounds since the time of her admission. From the time of the commencement of the defibrinated-blood treatment she steadily improved. She became able to eat fish and a light ordinary diet without discomfort; but the most striking fact was the diminution in size of the tumour, which practically disappeared. As in the former case, after reaching a climax there was a recrudescence of the symptoms, and some loss in weight, but the tumour did not return. The defibrinated blood was omitted on July 20, when she weighed 95 lb. Subsequently she mended, and left the hospital on August 9 considerably better and weighing 100 lb. There was undoubtedly some real improvement in this case, and the temporary relapse depended to some extent on fermentative changes taking place in the dilated stomach.

Several other cases have been treated with the defibrinated blood, and in some of them there has been apparent benefit, although others (a case of very advanced cancer of the liver, and one of peritoneal cancer) have not shown improvement.

In addition to the rapid reduction in size of the

growths in these two cases, a striking point was the improvement in the general symptoms and appearance of the two patients. Their cachexiæ practically disappeared, they became cheerful and seemed to get younger. In the first case the disappearance of the œdema of the legs was most remarkable, and never before had we seen cases of carcinoma, which had been bed-ridden for months previously and condemned by surgeons as being inoperable, become able to be up and about *apparently* vastly improved in health. It must be distinctly understood, however, that we do not assert that this treatment is in any way a cure for the disease. As mentioned at the outset of the description of the cases, we have no absolute proof that they were cases of carcinoma, and it must be remembered that spontaneous improvement and cure in some cases of cancer have undoubtedly occurred without any treatment whatever. Gaylord and Clowes have collected a series of these cases.<sup>1</sup> Moreover, we have been able to deal with only a few cases, and they have been under observation for only six months; therefore we cannot say whether the results are going to be permanent or even maintained for any length of time.

The reason why these cases are described is that they suggested to me a possible way in which the crucial experiment, mentioned at the end of the last chapter, could be carried out. It appeared reasonable that if one can cause the reduction in the size of a growth with

<sup>1</sup> *Seventh Annual Report* (Cancer Laboratory, New York State Department of Health).

amelioration of symptoms by general treatment, one might also be able to improve an accessible growth by locally inducing the proliferation of healing in it. If this were possible, and if a local, inoperable, broken-down scirrhus could be so improved by local treatment as to replace some of the infiltrating cells by normal ones, I considered that I should be justified then in carrying out the crucial test on these normal cells, and try to reinduce the abnormal infiltration amongst them once more by the direct application of auxetics and choline. In other words, if a case already has a large "inoperable" tumor and one is able to convert by treatment a portion of it into normal tissue, it would be useful to try temporarily to reconvert the normal tissue back into original condition in order to prove the main point of our researches. There would already be a neoplasm, and I proposed thus to test our theory in *in vivo* on a portion of it.

A patient suffered from an inoperable, fungating scirrhus of the breast. The ulcerated surface was about four inches in diameter. The edges were precipitous and excavated, and the whole appearance of the ulcer was typical of carcinoma. The surface was practically devoid of granulation tissue, and sections of it clearly showed its nature (figs. 119, 120). A portion of the surface, *i.e.* about a third of it, was scarified and globin was applied by being "dotted" over it (fig. 121). The remaining part of the ulcerated surface was untreated. No dressings were applied. This ulcer had a remarkable propensity for suppurating. No matter what was



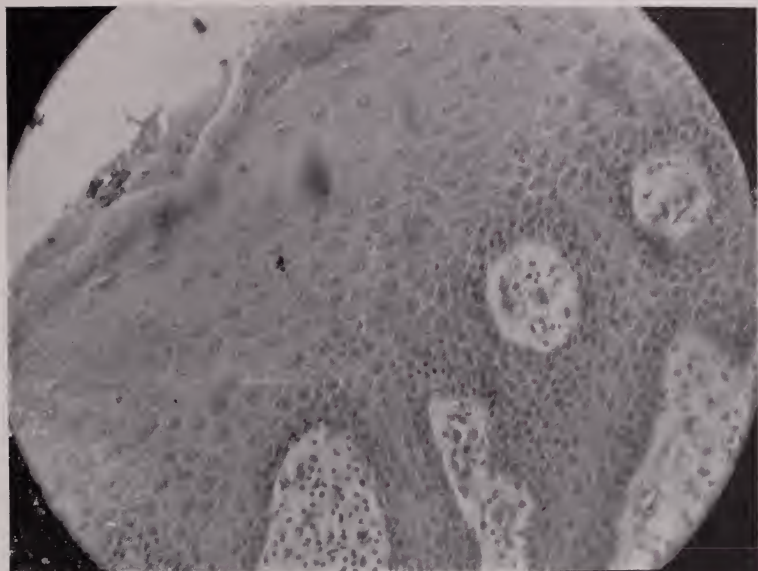


FIG. 119.—Section from the case of scirrhus of the breast. Low power.

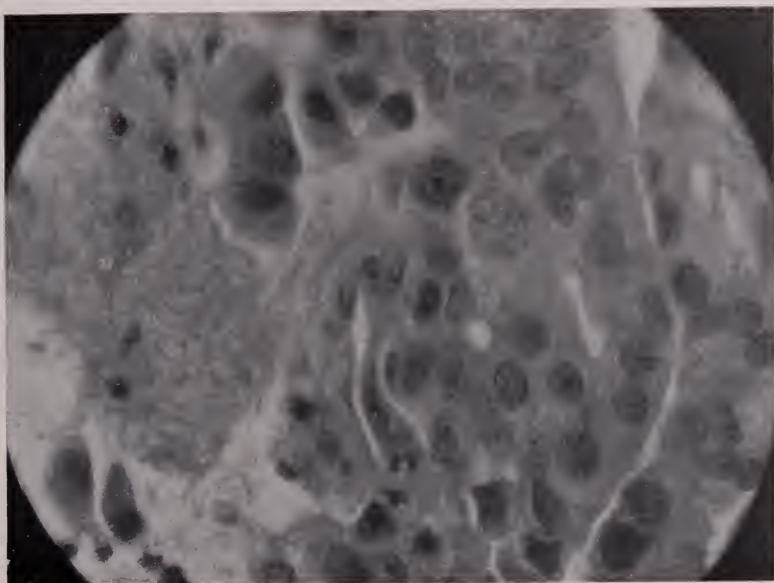


FIG. 120.—The same as 119. High power.







FIG. 121.—To show the way in which globin was “dotted” on to a portion of the malignant ulcer.



done to try to keep it "clean," pus quickly formed on its surface—much more quickly than it did on the benign callous ulcers which were also being treated. The result was that the scab formed by the globin very quickly broke down. When this occurred, the scab was removed by fomentations and the ulcer cleaned up as much as possible. Then the globin treatment was repeated, but it was always applied to the same portion of the ulcer. The other portion never received an application. This was repeated many times.

The improvement in the treated portion was gradual, but it was marked. The precipitous edges appeared to soften and become flattened. The base no longer suppurated in a few hours, and the suppuration was practically confined to the untreated portion. The glistening malignant surface of the treated portion gradually gave place to granulation tissue, and after about a fortnight's treatment there was a contrast between the treated and untreated portions of the broken-down surface. A portion of the treated part of the ulcer was now removed and sections cut from it, which show that the abnormal cells were now giving place to normal granulation tissue.

The treatment was continued once more, two parts of kreatin now being added to five parts of globin, and soon it was seen that the treated portion became softer, and the ulcerated edge ceased to extend. Another section was then cut, which showed that that part of the ulcer now seemed to be devoid of abnormal infiltration (figs. 122, 123).

I considered that the opportunity had now arrived to try the crucial test. A mixture was made of a solution containing five parts of globin and one part of choline. It was evaporated to dryness with aseptic precautions, and the dried mixture sealed up in a glass tube. A minute portion of the edge of the treated ulcer, from which the last section had been taken, was now scarified and small pieces of the dried aseptic mixture of globin and choline directly applied to it. In 48 hours a conical excrescence appeared at the seat of application. A section has been cut from it, and the photomicrograph shows apparently new malignant cells to be infiltrating the granulation tissue once more in an abnormal manner similar in appearance to the original infiltration of the ulcer (figs. 124, 125).

Now, this test is by no means conclusive. I cannot assert that there were no original carcinomatous cells at the seat of application, and that I was not merely producing augmented proliferation of these cancer-cells. The section may be fallacious owing to the "error of random sampling," for because no abnormal cells appear in samples removed from the treated site it does not prove that none exist in the neighbourhood. Still, the experiment is interesting, because the conical excrescence only appeared at the site of the application of the combined auxetic and the alkaloid of putrefaction, the rest of the ulcer remaining *in statu quo*.

This test will have to be repeated many times before one can speak conclusively on the subject;

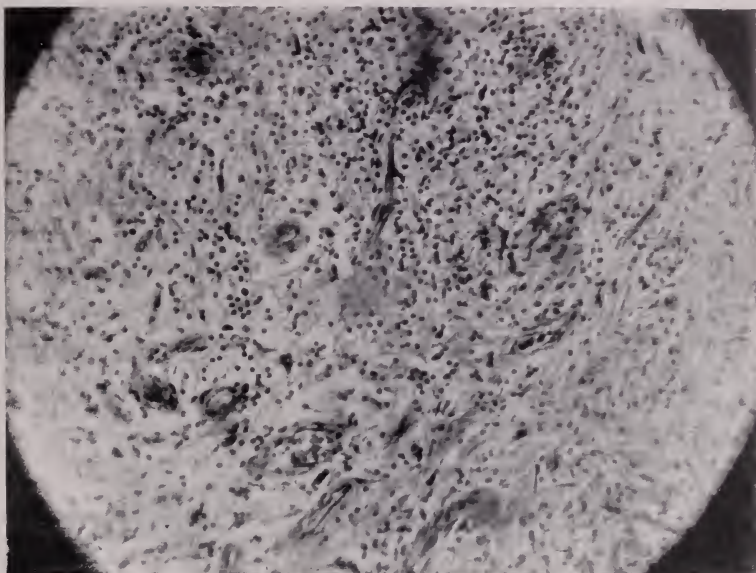


FIG. 122.—Section of a portion of the ulcer after treatment. Low power.

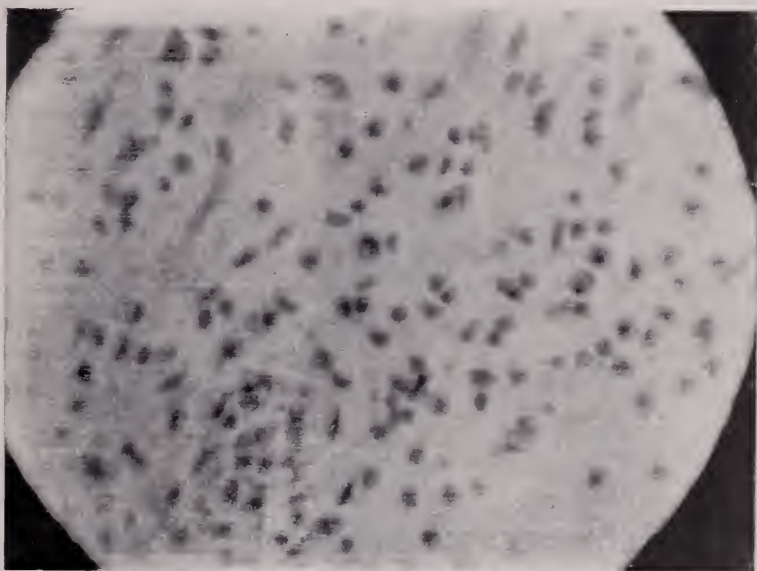


FIG. 123.—The same as 122. High power.





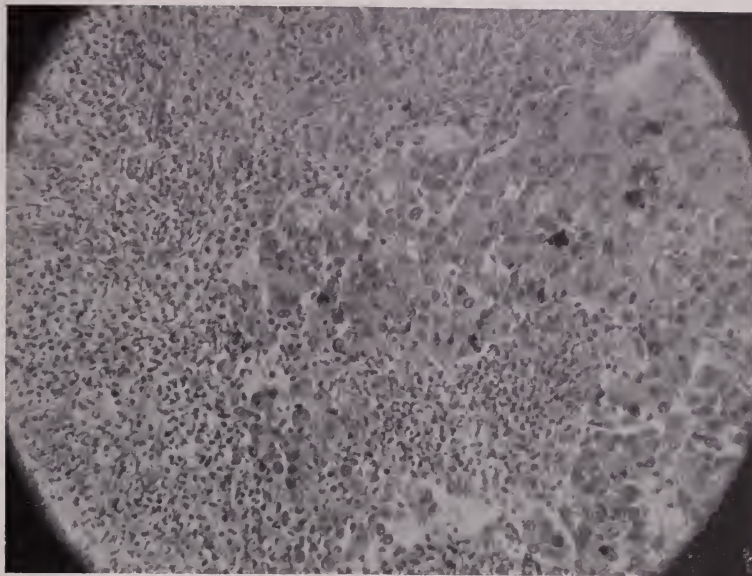


FIG. 124.—Section of the treated portion of the ulcer after the application of globin augmented by choline, showing reinfiltration. Low power.

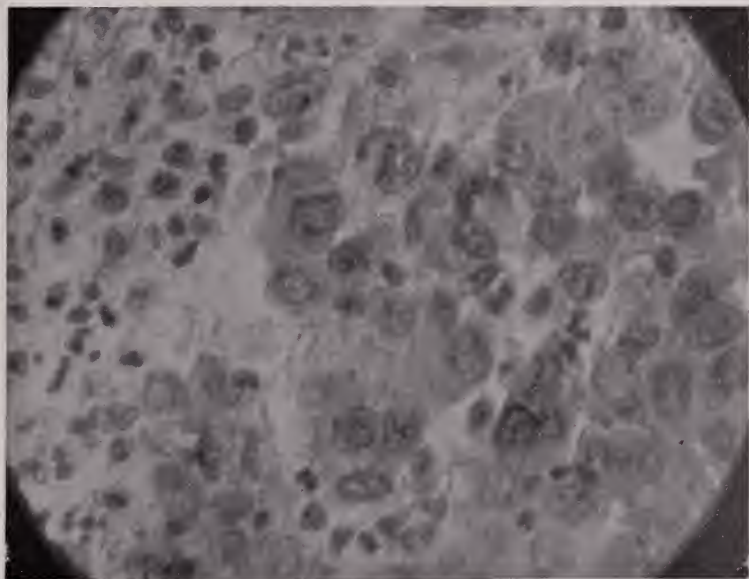


FIG. 125.—The same as 124. High power.



but from the general appearance of the ulcer, as well as from the microscopical section of it, we certainly think that it was the mixture of globin and choline which caused the reinfiltration. The application of globin alone to the edges of the sore merely induced the appearance of granulation tissue, and one would think that if a mixture of globin and choline does not cause carcinoma, it would merely have produced augmented proliferation of the healing cells with more granulation tissue; but apparently a new infiltration of epithelial cells appeared. I hope to be able to repeat this test under more favourable conditions.

The whole ulcer is now being treated with a mixture of globin and kreatin, and, although the edges of it are extending in some places, there can be no doubt that, on the surface at least, malignant cells are being replaced by normal granulation tissue. The whole growth is now comparatively freely movable, and it does not discharge profusely as it did. The patient no longer complains of pain, and, except for the extending edges, her general condition has greatly improved.

As it is possible that carcinoma may be due to the causes described in this book, and since the general treatment by defibrinated blood per rectum and the local treatment by globin and kreatin seem to have been followed by improvement, we think that the former might be tried to prevent recurrence after removal of a growth. Unfortunately we are not in a position to try this experiment, as early

cases which have been operated on do not come to our notice; we therefore take the liberty of suggesting that the prevention of recurrence might be undertaken by those who can watch a series of these cases which have been operated on. We cannot say, of course, whether recurrence will be prevented by rectal injections of defibrinated blood, but the treatment is harmless and it appears to be worthy of a trial.

If cancer is due to the causes which we think it to be, the reason for recurrence after removal of the original growth, which occurs in some cases in the operation scar, is open to two explanations: (1) that it may be due to portions of the original growth which have not been removed, and (2) that the healing site, although healing occurs by first intention, is a fruitful source of auxetics, and that the operation wound may easily become infected by the putrefactive organisms during the removal of the original growths. As already pointed out, certain putrefactive bacteria do not necessarily also cause suppuration; and therefore recurrence in the scar may be due to a fresh attack of cancer there. The proliferation of healing (even in a site healing by first intention) probably continues for weeks if not for months after the injury, because the initial proliferation increases the number of deaths, and possibly it is a long time before normal elimination is sufficiently restored to put a stop to the abnormal proliferation.

One frequently hears of cases in which "recurrence" takes place perhaps ten years after removal of the original growth. This must be due to a fresh attack of cancer. One of the commonest sites for it is in the

rectum, the very place where one would expect it to occur. Now that we know the causes of cell-proliferation it is difficult if not impossible to believe that a metastatic growth could remain malignant and quiescent for ten years without proliferating. We think that the increase of "restraining body" conferred on a person by a malignant growth may not last very long, and this, coupled with advancement of old age and possibly the existence of physiological excess of general auxetics which may occur in some persons, may predispose them to subsequent attacks of cancer. Our argument is that cancer is due to a combination of physiological auxetics and pathological alkaloids of putrefaction. The combination must be a definite one, or it will not be effectual; it must diffuse into the cells to a certain extent for a certain length of time, with due regard to the coefficient of diffusion of the cells; and lastly the vitality of the cells themselves must not be greatly impaired. We think that unless all these factors are in correct combination, malignant disease cannot occur.

With regard to the cause of sarcoma, we think that it is probable that the auxetic chiefly concerned in that disease is that contained in globin. Several surgeons have kindly informed us that in almost every case of sarcoma which they have seen there is a history of injury; and it is remarkable that sarcoma occurs most frequently in those tissues which are rich in hæmoglobin, namely, the choroid coat of the eye (melanotic sarcoma), the bone marrow, and the neighbourhood of muscles. The suggestion that globin is the source of the auxetic in sarcoma will explain the age-incidence



of the disease; for it probably only follows injury to large numbers of red cells. The length of life of red cells in the body is supposed to be only a matter of a few weeks, so that their anabolism and katabolism is continuous, and may not depend at all on the age of the person. Hence sarcoma may occur at any age.

Whether the alkaloids of putrefaction are concerned in sarcoma or not, we are not in a position to state, but interesting cases have been reported from time to time which were associated with suppurative foci. Quite recently a case was described in *The British Medical Journal*,<sup>1</sup> of an infant which had been injured in the neck by forceps at birth. Sarcoma followed on the injury, which was also complicated by suppurative otitis media.

The possibility of the alkaloids in both sarcoma and carcinoma being of the nature of leucomaines which are supposed to be absorbed from the intestines must not be forgotten.

The proliferation of leucocytes and lymphocytes in the leukæmias are also doubtless due to auxetics. Whether these diseases are caused primarily by injury to the spleen or not we do not know, but it is possible that this starts the proliferation. The spleen tissue has direct access, by means of the vessels, with the peripheral circulation, and presumably this is the reason for the leucocytosis and lymphocytosis in leukæmia. It is impossible to say whether the proliferation of leukæmia is of the augmented type, or whether an

<sup>1</sup> "A Case of Sarcoma of the Pectrous Bone," by W. H. Bowen and H. B. Carlyle (*B.M.J.*, June 25, 1910.)

alkaloid is present; but we may recall the interesting fact mentioned by Buchanan in his admirable book on the clinical pathology of the blood,<sup>1</sup> that he had noticed the discard of granules (flagellation) in the cells of cases of leukæmia. Possibly the leukæmias may be associated with the auxetic contained in globin, for the spleen is a very vascular organ; and if so, it may ultimately be found that leukæmia is a from of sarcoma of the spleen.

In concluding these descriptions of the researches which we have been able to carry out to the end of the first year and a half of the establishment of the Research Department of the Royal Southern Hospital at Liverpool, I wish once more to acknowledge my indebtedness to all those who have helped me so materially. I think that the new methods at our disposal have been the means certainly of solving the problem of the cause of normal human cell-division, and possibly, if not probably, of the cause of malignant cell-proliferation also. Much work remains to be done, however, some of which has already been started.

A series of more than ten "inoperable" cases of cancer are now being treated by defibrinated blood and by the local application of normal auxetics. Experimentation is begun to ascertain what organisms produce substances which "augment" the action of auxetics. The strength of the body in normal serum which restrains cell-division is being measured with a view

<sup>1</sup>R. J. Buchanan, *The Blood in Health and Disease* (Oxford Medical Publication).

to see if it varies in different persons, both in normal and in pathological conditions. The lengths of the lives of leucocytes are being measured in the presence of various strengths of auxetics and alkaloids of putrefaction. Many fields of work are now opened by the knowledge that the reproduction and multiplication of the cells of our bodies are due to certain known (and some as yet unknown) chemical agents. The knowledge that "healing" itself is caused by these agents may ultimately assist the medical man in his work, and I think that it will be found that trypanosomes amoebæ (the causes of dysentery), and other parasites also multiply in the body in response to the remains of dead cells. These paths of research will require many workers, and I am sure that their investigation will not be wasted.

Whether the benefit derived from the treatment adopted in two of the cases of cancer will prove to be of practical value or not remains to be seen. In any case it is capable of elaboration and further investigation. Even if it confers the smallest amelioration of symptoms, which it undoubtedly appears to have done in these two cases, something has been accomplished; but whether the benefit is lasting or not time alone will show.

# APPENDIX I

## ENUMERATION OF THE NUMBER OF GRANULES CONTAINED IN EOSINOPHILE LEUCOCYTES

TABLE I<sup>1</sup>

CONTROLS (healthy and diseases other than cancer). *Males*

Name.	Age.	Disease (or Health).	Number of Cells Counted.	Total Number of Granules Counted.	Number of Granules in Smallest Cell.	Number of Granules in Largest Cell.	Average Number of Granules per Cell.
Connolly . . .	12	Chorea . . . . .	5	811	142	175	162
Parker . . . .	14	Healthy . . . . .	1	174	...	...	174
Edwin . . . .	15	Healthy . . . . .	1	134	...	...	134
Brewer . . . .	15	Mitral Disease . . . . .	1	180	...	...	180
Hughes . . . .	15	Osteo-arthritis . . . . .	2	536	266	270	268
Mattison . . .	17	Malaria . . . . .	5	779	135	185	156
Duncan . . . .	20	Filariasis . . . . .	2	291	141	150	145
Bradley . . . .	21	Pneumonia . . . . .	4	561	124	147	140
Holding . . . .	22	Healthy . . . . .	2	354	177	177	177
McDonald . . .	24	Fracture . . . . .	1	227	...	...	227
Stevens . . . .	24	Sarcoma . . . . .	2	368	169	199	184
Ketch . . . . .	25	Varicocele . . . . .	1	155	...	...	155
May . . . . .	25	Fracture . . . . .	3	738	204	287	246
Ball . . . . .	25	Pneumonia . . . . .	3	502	101	220	167
Armstrong . . .	26	Sleeping Sickness . . . . .	1	82	...	...	82
Mahoney . . . .	26	Sarcoma . . . . .	1	175	...	...	175
Berry . . . . .	27	Floating Kidney . . . . .	2	475	197	278	237
Hankinson . . .	28	Acute Nephritis . . . . .	5	798	125	200	160
Cropper . . . .	28	Healthy . . . . .	21	3,390	114	260	161
McConnell . . .	32	Fracture . . . . .	5	745	127	165	149
Grue . . . . .	34	Hernia . . . . .	2	325	146	179	162
Jones . . . . .	34	Healthy . . . . .	4	769	155	208	192
Ross . . . . .	34	Healthy . . . . .	7	1,086	127	190	155
Smith . . . . .	36	Fracture . . . . .	2	297	105	192	148
Ritchie . . . .	46	Hernia . . . . .	5	748	122	192	150
Morgan . . . .	47	Hydrocele . . . . .	5	840	137	206	168
Daulby . . . .	50	Empyema . . . . .	2	345	151	194	172
Noble . . . . .	52	Addison's Disease . . . . .	6	875	124	190	146
Braig . . . . .	60	Stricture . . . . .	2	318	145	173	159
Cann . . . . .	62	Varicose Ulcer . . . . .	2	492	230	262	246
Gould . . . . .	65	Healthy . . . . .	2	336	147	189	168
Lowry . . . . .	66	Chronic Rheumatism . . . . .	1	165	...	...	165
Benn . . . . .	86	Healthy . . . . .	1	211	...	...	211
TOTAL . . . . .			109	18,282	...	Average	168

<sup>1</sup> In the averages fractions have been neglected throughout.

TABLE II

CONTROLS (healthy and diseases other than cancer). *Females*

Name.	Age.	Disease (or Health).	Number of Cells Counted.	Total Number of Granules Counted.	Number of Granules in Smallest Cell.	Number of Granules in Largest Cell.	Average Number of Granules per Cell.
Shankayne .	13	Chorea . . . . .	5	890	155	199	178
Frost . . . . .	16	Peritonitis . . . . .	6	957	117	212	159
Matthews . .	17	Hysteria . . . . .	1	117	...	...	117
Farrington .	19	Healthy . . . . .	2	334	155	179	167
Simpson . . .	21	Osteo-arthritis . . . .	1	136	...	...	136
Stone . . . . .	22	Chlorosis . . . . .	6	994	146	201	166
Francis . . . .	24	Healthy . . . . .	2	412	168	244	206
Baker . . . . .	27	Lymphadenoma . . . .	2	365	172	193	182
McKey . . . .	35	Carbuncle . . . . .	1	201	...	...	201
Jackson . . .	38	Myxoma . . . . .	2	338	163	175	169
Harris . . . . .	56	Healthy . . . . .	2	397	172	225	198
Swalwell . . .	56	Osteo-arthritis . . . .	5	801	120	192	160
Wilson . . . .	65	Hernia . . . . .	5	724	127	165	145
Benn . . . . .	90	Healthy . . . . .	2	390	186	204	195
		TOTAL . . . . .	42	7,056	...	Average	168

TABLE III  
CANCER CASES

A. *Males*—

Name.	Age.	Locality of Disease.	Number of Cells Counted.	Total Number of Granules Counted.	Number of Granules in Smallest Cell.	Number of Granules in Largest Cell.	Average Number of Granules per Cell.
Doyle . . . . .	32	Stomach . . . . .	5	699	113	166	140
Mackie . . . . .	32	Lung (Secondary) . .	2	340	129	211	170
Rhead . . . . .	34	Testicle . . . . .	7	1,036	104	187	148
Ya Foo . . . . .	44	Penis . . . . .	7	1,201	132	197	171
Nesborough . . .	44	Lip . . . . .	3	468	132	199	156
Donahern . . . .	59	Sigmoid . . . . .	6	835	106	175	139
Gardiner . . . . .	59	Stomach . . . . .	1	150	...	...	150
Welsh . . . . .	65	Penis . . . . .	6	1,013	132	196	169
Whelan . . . . .	68	Tongue . . . . .	5	928	140	234	186
TOTAL . . . . .			42	6,670	...	Average	159

B. *Females*—

Duncan . . . . .	35	Stomach . . . . .	2	357	156	201	178
McCann . . . . .	41	Liver . . . . .	2	323	121	202	161
Evans . . . . .	42	Breast . . . . .	1	144	...	...	144
McQuillian . . .	42	Uterus . . . . .	1	217	...	...	217
Griffiths . . . . .	45	Breast and Pelvis . .	6	1,201	163	273	200
Jones . . . . .	45	Breast . . . . .	1	119	...	...	119
Hiles . . . . .	49	Breast . . . . .	2	313	146	167	156
Walker . . . . .	54	Stomach . . . . .	5	767	147	158	153
Griffiths . . . . .	56	Cervix Uteri . . . . .	5	953	121	247	191
Griffiths . . . . .	56	Breast . . . . .	7	1,046	109	197	150
Roberts . . . . .	56	Stomach . . . . .	3	387	96	159	129
Hall . . . . .	56	Breast . . . . .	1	120	...	...	120
Cunning . . . . .	66	Breast . . . . .	6	804	88	158	134
TOTAL . . . . .			42	6,751	...	Average	161



TABLE IV

AVERAGE NUMBER OF GRANULES IN (A) LARGEST, AND (B) SMALLEST CELLS

1. *Males and Females separated—*

	Controls. Males. Table I.	Controls. Females. Table II.	Cancer. Males. Table III.	Cancer. Females. Table III.
(A) AVERAGE number of granules in largest cells	204	199	196	196
(B) AVERAGE number of granules in smallest cells	150	153	124	127

2. *Males and Females combined—*

	Average Number of Granules in Largest Cells.	Average Number of Granules in Smallest Cells.
Controls . . . . .	202	151
Cancer . . . . .	196	126

It should be noted that the greatest difference between Cancer and Control cells is in the smallest leucocytes.

## SUMMARY

Total number of persons examined .....	69
Total number of cells photographed .....	235
Total number of granules counted .....	38,759

*Table showing differences between the cells of Control (healthy and diseases other than cancer) persons and Cancer persons*

	Persons Examined.	Cells Photographed.	Granules Counted.	Average Granules per Cell.
Controls.....	47	151	25,338	168
Cancer .....	22	84	13,421	160

*Table showing Influence of Sex**Males—*

	Persons Examined.	Cells Photographed.	Granules Counted.	Average Granules per Cell.
Controls.....	33	109	18,282	168
Cancer .....	9	42	6,670	159

*Females—*

	Persons Examined.	Cells Photographed.	Granules Counted.	Average Granules per Cell.
Controls.....	14	42	7,056	168
Cancer .....	13	42	6,750	161

Number of granules in smallest cell, 82. Number in largest cell, 287.

*Variation in the number of granules contained in the cells of one person*

21 cells from Cropper. Smallest cell contained 114 granules; largest contained 260. The average number of granules in the 21 cells is 161.

## APPENDIX II<sup>1</sup>

### SOME COMPARATIVE MEASUREMENTS OF THE LIVES OF LEUCOCYTES<sup>2</sup> WHEN THE CELLS ARE RESTING IN THE PLASMATA OF DIFFERENT PERSONS

#### AND THE POSSIBLE APPLICATION OF SUCH MEASUREMENTS AS AN AID TO DIAGNOSIS IN INFECTIVE DISEASE

OF recent years I have been endeavouring to ascertain the effect produced by one person's plasma on the life of another person's leucocytes. It appeared reasonable to suppose that the plasma of a person suffering from an infective disease would be poisonous to the leucocytes of healthy persons. If this is the case it might also be reasonable to suppose that the same plasma would not be so poisonous to the leucocytes of another person suffering from the same disease, because it is probable that the cells would be already used to, or immune against, the toxin; and furthermore that if the toxin of one infective disease differs from the toxin of another infective disease, it might be inferred that an immunity on the part of a leucocyte against one disease will not render it immune against another. Therefore, provided it is possible to tell accurately when a leucocyte is dead—that is, if one can differentiate a living

<sup>1</sup> A method for estimating the number of living and dead leucocytes contained in a given sample of blood; and another convenient formula for the preparation of "kinetic jelly." Being a paper reprinted from *The Lancet* of February 6, 1909, by kind permission of the and Editor of that Journal.

<sup>2</sup> The word "leucocyte" refers to the neutrophile polymorphonuclear leucocyte.

from a dead cell—it also will become possible to measure the lengths of the lives of leucocytes after they have been removed from the body. And this will enable us to make comparative measurements of the lives of leucocytes when they are mixed with the plasmata of different persons. Supposing, therefore, it is true that an infected plasma shortens the lives of a healthy person's leucocytes but does not shorten the lives of the leucocytes of another person suffering from the same disease, it may be useful to reverse the process and assist in the diagnosis of infective disease by making measurements of the lives of such a patient's leucocytes when they are mixed with different plasmata. For instance, if the leucocytes of a person suffering from an indefinite infective disease are found to be easily killed by the plasmata of persons suffering from a variety of diseases, but are not comparatively easily killed by the plasma of a person suffering from, say, typhoid fever, it might be inferred that the patient is suffering from, or has recently suffered from, typhoid fever, because his leucocytes are used to, or immune against, that disease.

The above is the enunciation of a problem which I set myself to solve several years ago, and this paper describes the experiments which have been conducted to investigate the last part of it—*i.e.* with the object of determining the actual measurements of the lives of leucocytes when they are placed in the plasmata of people who are suffering from various diseases. The earlier researches made in order to differentiate living from dead leucocytes have already been published in the *Journal of Physiology* (1),<sup>1</sup> and the actual method employed to estimate how many living and how many dead cells there may be in a given volume of citrated blood has been described in *The Lancet* of January 16, 1909 (2). This method may be again briefly summarised thus:

*Method for counting the number of living and dead leucocytes in a given sample of citrated blood.*—The following solutions are prepared and a jelly is made from them. 1. A volume of Unna's polychrome methylene blue (Grübler) is diluted with two volumes of water. 2. A solution containing 2 per cent of agar in water,

<sup>1</sup> The figures within parentheses refer to the bibliography at the end of the article.

filtered and sterilised. 3. An accurately neutralised solution containing 4.5 per cent sodium citrate, 1.5 per cent sodium chloride, and 0.225 per cent atropine sulphate. 4. A 5-per-cent solution of sodium bicarbonate. In a test-tube mix one cubic centimetre of the diluted stain, two cubic centimetres of the citrate solution, and three cubic centimetres of the molten agar solution. To this mixture a quantity of the alkaline sodium bicarbonate solution must be added in order to cause the excitant for leucocytes contained in the jelly to diffuse into the cells, and the quantity added varies with the temperature of the room.<sup>1</sup> If measurements are going to be made in a room with a temperature of between 60° and 70° F., about 0.25 cubic centimetre of the alkaline solution should be added. The mixture is then boiled until it froths up the tube and a drop poured on to a slide and allowed to set so as to form a film. Supposing a given capillary tube contains the blood-corpuscles of one person mixed with the plasma of another, the average number of living and dead leucocytes in the tube can be estimated by placing a drop of its contents on a cover-glass which is inverted and allowed to fall on the agar film. After two or three minutes the granules but not the nuclei of the living leucocytes will stain and those cells will show exaggerated amoeboid movements, whereas the dead cells will remain immobile. Moreover, the dead cells may be achromatic (3), in which case they will not stain. Their nuclei may appear as a single nuclear mass, or their nuclei may even stain, or the dead cells may have undergone other changes which have been described in former papers (1, 2). Field after field should be rapidly passed in front of a 1-6th inch or equivalent objective and the number of the living and dead cells counted. Several preparations can be rapidly examined and an average struck so as to give an estimate of the number of living and dead cells in the given capillary tube. No difficulty is met with in making the counts, for living can be readily differentiated from dead cells by the presence or absence of exaggerated movements.

If all the leucocytes appear to be dead, and especially if the agar jelly has not previously been tested, it is as well to control

<sup>1</sup> A scale has been given in the former paper.

the measurement—that is, to see that the jelly will actually excite living cells—by placing a drop of fresh citrated blood on to another part of the same film and noting whether stimulated movements of all the leucocytes occur.

*Procedure for the preparation of capillary tubes containing the plasma of one person and the leucocytes of another.*—It will simplify description if the details of sterilisation and the precautions for ensuring asepsis are omitted. Since the presence of bacteria shortens the lives of leucocytes (2) it is obvious that aseptic precautions are essential, but the details for sterilisation are so well known that they need hardly be repeated. A capillary tube of glass is prepared which has such a diameter that blood will run into it by capillarity and at the same time its flow can be controlled by gravity. I use a tube with a lumen of about two millimetres. 15 portions equal to each other are marked off with a pencil. The marks begin at one end of the tube which is zero, but the tube is at least two inches longer than mark 15. The portions are rendered equal by calibration with mercury, and although the length of each is immaterial, I have found that about half a centimetre is a convenient length for practical purposes and I use a tube about 13 centimeters long. A neutral solution is made which contains 3 per cent of sodium citrate and 1 per cent of sodium chloride. Some of this is drawn up into the tube until its upper limit or meniscus stands at mark 6. Blood from the finger of the person whose plasma is going to be tested is added until the meniscus stands at mark 12, care being taken that no bubble of air separates the two fluids. Mixture is carried out by allowing the two fluids to gravitate up and down the tube six times. The tube is sealed and centrifugalised; the blood being driven towards zero. The end remote from zero is then unsealed and the portion containing the precipitated corpuscles is separated and discarded by cutting the tube at 4. Eight portions of the tube now contain citrated plasma. If, owing to the sealing process, much of the tube has been occluded at zero the upper meniscus may stand above mark 12. This can be corrected by tapping out the excess of fluid on to a sterile slide, controlling the amount removed by the finger on



the end remote from the mark 4. The lower meniscus standing at 4 where the tube has been cut, and the upper meniscus standing at 12, blood from the finger of the persons whose corpuscles are going to be tested is added until the upper meniscus stands at 13 (*i.e.* the mixture equals 1-9). Mixture is ensured as before and the tube sealed. It will be seen that although the tube contains the plasma of both persons the corpuscles are bathed in a solution containing four times as much plasma of the first person as of the second. A series of tubes may thus be made.

*Appliance to ensure continual mixture and to prevent the corpuscles from adhering to the glass.*—If a capillary tube prepared in the way which has been described is laid on a flat surface, the corpuscles will soon gravitate to the most dependent side and will ultimately adhere to the glass. The following appliance prevents this. By means of a simple clockwork movement a split drum is made to revolve once in about three minutes. The drum is so adapted that the mouth of a long test-tube (having a diameter of one centimetre and the cavity of which is lined with a roll of blotting paper) fits accurately on to it and revolves with it. The apparatus is so arranged that the tube is horizontal and is of such a size that it can be placed in the incubator if necessary. The capillary tubes inserted into the test-tube are continually tumbling over each other by gravity as the test-tube revolves, and in so doing revolve themselves. The blood-cells in their turn are continually gravitating in different directions through the citrated plasmata. It has been found that this device prevents them adhering to the glass and ensures them being evenly distributed through the citrated plasmata provided the ends of the capillary tubes are not bent over when sealed. This apparatus also insures all capillary tubes being subjected to the same conditions of temperature.

*Procedure for measuring the lives of the leucocytes contained in the tubes.*—Samples of the contents of the capillary tubes are examined on stimulating agar by the method already described. If all the cells are alive the tubes are resealed and returned to the revolving apparatus to be examined later, and so on. By this means the percentage of living and dead cells in a tube can be

estimated. It is important to remember that in striking these averages only an approximate estimate can be obtained, and that therefore the greater the number of tubes made the better, as the error decreases with the greater number of leucocytes counted. In the experiments which I am about to record I have counted about 500 leucocytes in each case by making five films from each of five tubes, and counting about 20 leucocytes in each film. Since it is obvious that the greatest error may occur when the number of living approximates the number of dead cells in a tube, the following experiments would appear not to be very erroneous, judging by the application of Poisson's formula, which shows that supposing there are half a million leucocytes in the five tubes, which is an excessive estimate, a count of 500 cells would give a possible error of not more than about 6 per cent, even when the numbers approximate.

Before enumerating the actual measurements there is yet another question to be considered, a point upon which I wish to lay great emphasis—namely, that all measurements of the lives of leucocytes should necessarily be comparative. For instance, it would be fallacious to say that a typhoid plasma killed a person's leucocytes more rapidly than a septicæmic patient's plasma, when the typhoid measurement was made to-day and the septicæmic measurement made three days ago; for even if there was a great difference in the length of the lives and the same person's leucocytes were used, one cannot say that that person's leucocytes were in the same state to-day as they were three days ago, although the person is apparently in the same healthy condition.

Again, I have shown (4) that the factor *heat* in accelerating the diffusion of substances into cells also materially affects the lives of the leucocytes, since the cells are necessarily resting in a citrate solution which is itself poisonous to some extent, and even the temperature of incubators is variable. It is thus of the utmost importance that when the lives of a person's leucocytes, which have been placed in the plasma of a person suffering from an infective disease, are measured, a simultaneous measurement of the same leucocytes shed at the same time must be made in the plasma of a healthy person. And it is only by the difference between the

two that the result can be determined. In other words, all measurements must be simultaneously controlled by other measurements and the contrast is the result. It is also obvious that since heat and the citrate solution both affect the lives of the cells, all tubes, whether containing infected or control plasma, must be subjected to the same conditions as regards temperature. And it is essential that the same citrate solution must be employed both for the test and the control. Unless these essential details are adhered to, any measurements may be considered to be worthless. Leucocytes are very sensitive to changes in temperature when they are resting in citrate solution, but if a change occurs and all tubes are subjected to the same change the contrast in the length of life holds good. The most favourable arrangement of the citrate solution has already been given. It should be quite neutral, because if alkaline it shortens the lives of the cells.

Leucocytes appear to live longest at about  $20^{\circ}$  C. They will not live very long at  $37^{\circ}$ , and at  $10^{\circ}$  will live longer than at  $37^{\circ}$  but not so long as at  $20^{\circ}$  C. I have already suggested (4) that this may be due to the accelerated absorption of the poisonous salts in the citrate solution caused by heat, and this will also explain the early death in the presence of alkali which also accelerates diffusion. I presume that the reason why they live longer at  $20^{\circ}$  than at  $10^{\circ}$  is because their normal temperature is about  $37^{\circ}$  C. and that they die in the cold in spite of the delayed absorption.

In the following experiments a temperature of  $30^{\circ}$  C. was employed with the specified citrate solution, and control experiments were conducted in each case, the results given being the difference in the measurements between the test and control.

### *Measurements*

*Length of the life of healthy person's leucocytes when resting in their own plasma.*—As has been shown in a former paper (2), an average shows that all the cells are alive in 24 hours; the majority are alive in 36 hours; about 50 per cent are dead in 48 hours; and all are dead in 86 hours.

*Healthy person's leucocytes; other healthy person's plasma.*—All cells were alive in 14 hours; about 50 per cent were dead in 18

hours; the majority were dead in 22 hours; and all were usually dead in 28 hours. The difference between these averages may be said to be about 30 hours. I conclude that the plasma of one person is poisonous to another person's leucocytes.

*Healthy person's leucocytes; plasma from cases of typhoid fever.*—All cells dead in 14 hours. Difference between test and control about six hours, which is the average out of four cases.

*Healthy person's leucocytes; plasma from cases of malaria.*—Majority of cells dead in 16 hours, a few alive in 18 hours. Occasionally 50 per cent were alive in 16 hours. Average difference between 12 cases and their controls about two hours.

*Healthy person's leucocytes; plasma from cases of phthisis.*—Majority dead in 17 hours. Average difference between five cases and their controls about one hour. Sometimes it was as much as four hours, but in very chronic cases there was little difference.

*Healthy person's leucocytes; plasma from a case of osteo-myelitis.*—50 per cent dead in 14 hours. Repeated with a case of gangenous appendicitis the films showed that the majority were dead in 14 hours. The difference between these cases and their controls were five hours and three and three-quarter hours respectively.

*Healthy person's leucocytes; plasma from a case of purpura hæmorrhagica.*—Majority dead in 15 hours; all dead in 20 hours. Difference from controls five hours.

*Healthy person's leucocytes; plasma from a case of chorea.*—All cells dead in 14 hours. Difference about six hours.

*Leucocytes from cases of typhoid fever; plasma from other cases of typhoid fever.*—Average from three groups of cases, all of which reacted to Widal's reaction and were in the third or fourth week of the disease except one which was convalescent. These groups include the cases mentioned above. There was never a difference of more than one and a half hours between the death of the majority of cells in test and control tubes.

*Leucocytes from cases of malaria; plasma from other cases of malaria.*—Five cases. The majority of cells in all cases were alive in 18 hours. Practically no difference from controls.

*Leucocytes from cases of phthisis; plasma from other cases of phthisis.*—Four experiments. 50 per cent dead was the average in 18 hours; very little difference from controls, sometimes the cells lived longer than in the controls.

*Leucocytes from cases of malaria; plasma from cases of typhoid fever.*—The majority of the cells in most instances were dead in 14 hours. Differences varied from four to six hours.

*Leucocytes from cases of typhoid fever; plasma from cases of malaria.*—About 50 per cent were usually dead in 16 hours and all were dead in 20 hours in all cases. Five cases tried; average difference about three hours.

*Healthy person's leucocytes; plasma from cases of carcinoma.*—Seven cases; all cells alive in 16 hours; a large number alive in 20 hours. Usually there was little difference between the effect of cancer plasma and that of a healthy person.

From the foregoing measurements it would appear that in the cases which have been experimented with the plasma of persons suffering from infective diseases is poisonous to a healthy person's leucocytes and to the leucocytes of another person suffering from another disease, but is not so poisonous to the leucocytes of another person suffering from the same disease. I submit that it may be reasonable to suppose that such may be the case in other infective diseases.

*Precautions.*—In comparing the lengths of the lives of leucocytes of persons suffering from chronic infective diseases both in another infected person's plasma and in healthy plasma, I have frequently found that such cells will not live so long as the cells of healthy persons subjected to the same conditions. This was further investigated by comparing the lives of leucocytes taken from cases of chronic illnesses in their own plasmata with the length of the lives of the cells of healthy persons in their own healthy plasmata. In cases of chronic phthisis, malaria, Hodgkin's disease,<sup>1</sup> etc., I have found that the leucocytes will not live even in their own plasma nearly so long as if they belonged to a healthy person, as much as

<sup>1</sup> It has been noticed that stain will diffuse more readily into the blood-cells of these patients—that is, that these diseases, and probably other chronic illnesses, cause a lowered "coefficient of diffusion" in blood-corpuscles.



a day's difference having been observed; and we may infer that these diseases, and probably others also, cause a loss of vitality in the patient's leucocytes, so that by this procedure the loss of vitality can be measured. It is important to remember this point, for if the making of a measurement is delayed it may be found that all the cells are dead in both control and test preparations. This method of measuring the lives of leucocytes may also prove of value in prognosis as well as in diagnosis.

I do not think that any difficulty will be met with in making the counts, with the exception of a possible one caused by the agglutination of the leucocytes. Occasionally large clumps are met with. If the cells are clumped, however, it does not necessarily follow that they are dead—far from it, for they may be very active, though I am of opinion that if clumped death will soon occur. The cells in a clump can generally be counted. Ruptured cells are counted as dead. If bacteria are seen in large numbers in a film the capillary tube is discarded. The revolving apparatus is not essential, but more constant results have been obtained by its use. As far as possible I have purposely avoided handling the blood of the person whose leucocytes are to be tested, for fear of injuring the cells. The variations of the alkalinity of the plasma may, I think, be neglected, as it is not sufficient materially to alter the length of the lives of the cells. This is borne out by the experiments with cancer plasma, because that plasma is more alkaline than normal and yet does not shorten life.

### *Summary*

I fear that it is too early to arrive at any definite conclusions from so small a number of experiments, but I think that there publication is justified in order to explain the method employed and because the results are sufficiently promising to warrant further investigation, though the work must still be regarded as being in the experimental stage. I hope that this method will be tried by others, as the problem given in the enunciation may lead to important developments, and especially as this kind of research involves the striking of averages and a large amount of experiment to



determine the points. The method may also be useful to others studying other branches of immunity. As I have already stated, my aim is to be able to assist in the diagnosis of infective disease by this method, but a large amount of material will be required before one can determine its value in this direction, and I have mentioned its possibilities with reference to prognosis. The stage in a disease in which measurable immunity appears in a leucocyte also remains to be determined.

To summarise the method by which I endeavour to assist in a diagnosis in a case of infective disease, a small quantity of blood from a patient is mixed with eight times its volume of the citrated plasma of other persons who are known to be suffering from certain infective diseases and also with the citrated plasma of a healthy person. For this last purpose I sometimes use my own plasma. The method has been described. The capillary tubes are kept together in the revolving apparatus for about 14 hours. Then some agar films are prepared from jelly which will excite movements in living leucocytes, and samples of the contents of the tubes are examined on these films. The number of living and dead cells are averaged, and the difference between the lengths of the lives of the cells when resting in healthy and infected plasmata are determined. When an infected plasma is found which will not comparatively shorten the lives of the patient's leucocytes, it seems probable that the patient is suffering from the same disease as the person from whom the plasma was taken. I generally confirm this procedure by reversing the process and trying the patient's plasma on the leucocytes of other persons suffering from the disease determined, taking care to make controls in this case as well as in the first by making measurements with healthy plasma and with the plasma of persons suffering from other diseases.

The method described in this paper has two disadvantages: first, in keeping the tubes at 30° C., and, secondly, in counting 500 leucocytes in each case, which is most tedious. The rest of the method takes very little time; collecting the plasmata and mixing them with the patient's corpuscles is soon accomplished, and when the tubes are in the revolving apparatus they require no further attention until the time has come to estimate the number

of living and dead cells in them. The agar jelly can be made from stock solutions as specified and kept in test-tubes for months, as moulds will not grow on it. Films are rapidly prepared by boiling the jelly in a tube in a spirit-lamp flame.

With regard to the two disadvantages, an incubator working at 30° C. is not usually within reach even in laboratories, although Hearson's apparatus will maintain this temperature if fitted with a special capsule. Since my aim is to make this possible diagnostic method suitable for practical purposes even away from the vicinity of a laboratory, I dispense with an incubator and employ the ordinary temperature of a room, say between 60° and 70° F. In order to do this the citrate solution is modified. If the solution already specified were used at such a temperature the leucocytes might live for a long time even in an infected plasma, and a day or two might elapse before sufficient deaths occurred among the cells to make a contrast. Consequently I deliberately shorten the life of the cells by using a solution containing 1.2-per-cent sodium citrate and 1-per-cent sodium chloride. As the same solution is employed for all tubes the artificial shortening of life does not appear to vitiate the results. There are several ways by which this shortening of life can be accomplished, though I consider the lowering of the sodium citrate content to be the most suitable. Using this solution it has been found that the majority of healthy cells in another healthy person's plasma are dead in about 24 hours if kept at the room temperature, which, of course, may be variable. So a contrast can usually be obtained within 24 hours of mixing the bloods. With regard to the second disadvantage, I hope by experiment to ascertain the minimum number of leucocytes which it may be necessary to count to obtain a trustworthy average. I am sure that a smaller number than 500 will be sufficient. I am also experimenting with a greater concentration of plasma with a view to obtaining a wider contrast between the length of the lives of cells in healthy and infected plasma.

In conclusion, I wish to suggest that this method may also be useful from a medico-legal aspect, for I have found the leucocytes alive in the blood removed from the hearts of bodies which have been lying in the mortuary for 24 hours or more, and it may be

possible to state how long a person has been dead by estimating the percentage of living cells so many hours after the death of the subject.

*Bibliography.*—H. C. Ross: (1) "On the Death of Leucocytes," *Journal of Physiology*, vol. xxxvii., 1908, p. 327; (2) "On a Combination of Substances which Excites Amœboid Movements in Leucocytes," *The Lancet*, Jan. 16, 1909, p. 152; (3) "On the Cause of Achromasia in Leucocytes," *The Lancet*, Jan. 23, 1909, p. 226; (4) "On the Modification of the Excitant for Leucocytes composed of Methylene Blue and Atropine," *The Lancet*, Jan. 30, 1909, p. 313.

## APPENDIX III

A METHOD BY WHICH CELLS CAN BE EXAMINED MICROSCOPICALLY  
BETWEEN A COVER-GLASS AND A JELLY-FILM WITHOUT  
THE FORMER EXERTING ANY PRESSURE ON THEM. (A  
“HANGING DROP” PREPARATION WITH THE JELLY METHOD)

Two round cover-glasses are required. One should have a diameter of half an inch, the other of seven-eighths of an inch. The jelly from which the film is to be prepared is boiled and a drop of it run on to a slide. Immediately, before the jelly has had time to set, the small cover-glass is allowed to fall flat on the centre of the jelly-film on the slide. Since the jelly is not set, the cover-glass sinks into but not actually through it. The film with the cover-glass embedded in it is allowed to set for about five minutes. One needle is then placed vertically against one edge of the small cover-glass embedded in the jelly, and the point of another needle is inserted under the opposite edge of the cover-glass. By a jerk of this needle the embedded cover-glass is lifted out of the jelly, when it will be found that a shallow circular depression exactly corresponding to the cover-glass is left in the jelly-film. The base and sides of the depression will, of course, be composed of jelly. The cells to be examined are placed in citrate solution on the large cover-glass, which is inverted and allowed to fall flat over the depression in the film. By this means the large cover-glass is resting on the raised sides of the depression, but the cells are in the depression. They can now be made to absorb substances from the jelly, but the cover-glass does not press them into it unless the cells are very large. This method is useful for the *in-vitro* staining of motile bacteria, trypanosomes, etc.

## APPENDIX IV

### A POSSIBLE ASSOCIATION BETWEEN THE AUXETICS OF HEALING AND IMMUNITY AGAINST INFECTIVE DISEASE

THE fact that auxetics contained in the remains of dead tissues and in globin will cause the cell-proliferation of healing has suggested a new line of research connected with the problem of immunity. Since the cell-proliferation of healing is caused by chemical agents, and since the actions of these agents can be augmented by substances produced by bacteria, and inhibited by normal serum, it may be useful to ascertain the action of disease germs on (1) the remains of dead tissues and globin, and the auxetic it contains, and (2) on serum. It is obvious that if disease-germs decompose auxetics, there will be less cell-proliferation of healing; but if they produce substances which augment the action of auxetics, or if they prevent the inhibitory action of serum, then they will tend to assist in healing an injury. Before any disease-germ can obtain a footing in the body it must cause an injury which is followed by an attempt at healing. If this healing is prevented, disease will be the result. If, however, healing occurs successfully, the patient will remain immune. Hence this suggests that the action of disease-germs on the *sources* of the causes of the cell-proliferation of healing should be investigated. In reality, the problem is a bacteriological one, but the investigation of it will not, I think, be very difficult.

## INDEX.

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- Achromasia, 4, 14, 21, 33, 43, 55  
 Acids, 71, 89  
 Aconitine, 149  
 Agar, 7, 9, 36, 40, 83  
 Alkalies, 71, 77, 86, 88  
 Alkaloids, 149, 157, 352  
 Amitotic divisions, 297  
*Amæba Coli*, 98, 400  
 Amœboid movements, 67  
 Apparatus, 18, 20, 27, 34, 410  
 Archoplasm, 112, 123  
 Artefacts, 17  
 Asymmetrical mitosis, 10, 12, 44,  
     132, 235, 240, 300  
 Augmentation of cell-division, 227,  
     310  
 Auxetics, 6, 232, 292  
 Azur dye, 2, 237, 347
- Bacillus subtilis*, 366  
 Bacterial toxins, 8  
 Basophile leucocyte, 96, 274, 281  
 Benign tumours, 7, 339, 340  
 Blood alkalinity in cancer, 176  
 Blood-platelets, 5, 102, 113, 117, 124  
 Blood-serum, 9, 374, 376  
 Brownian movements, 38, 39, 52  
 Brucine, 149
- Cadaverine, 350, 365  
 Callus, 336  
 Cancer, ætiology of, 161, 164, 360  
     age-incidence of, 161, 360  
     cases of, 379  
     causes of (theory), 360  
     climatic incidence of, 164, 371  
     place-incidence of, 371  
     plasma, 159  
     prevention of recurrence of, 395  
     recurrence of, 397  
     site-incidence of, 363  
 Capillary tubes, 18  
 Centrosomes of leucocytes, 258  
     of lymphocytes, 186
- Chemotaxis, 150  
 Choline, 352, 365  
 Chorea, 8  
 Chromosomes of leucocytes, 258  
     number of, 239  
     of lymphocytes, 186  
 Citrate solution, 41, 82  
 Citric acid, 85, 88  
 Cocaine, 149  
 Codeine, 149  
 Coefficient jelly, 85, *et seq.*  
 Coefficient of diffusion, 61, *et seq.*  
 Concentration of substances, 68  
 Cornea, 337  
 "Corns," 337  
 Crenation, 38  
 Crescent, malarial, 323  
 Cytogeny, causes of, 158, 166  
 Cytoplasm, 52, 59, 62, 82
- Death, delay of, 107  
     in cancer, 165, 366  
 Death-struggles, 147, 246, 346  
 Decrepitude, 163  
 Defibrinated blood, 378  
 Diffusion of substances, 67, *et seq.*  
     of two substances, 145  
     vacuoles, 103, 111  
 Doses of alkaloids, 148  
 Drugs, 61, 63
- Elimination, 338  
 Embryo, 7  
 Eosinophile granules, 274, 281, 401  
     leucocytes, 94, 293  
 Epithelial cells, 73, 352  
 Erythrocytes, 72  
 Examination of specimen, 102  
 Excess of diffusion, 103  
 Excitation, 130  
 "Experimental ten minutes," 249  
 Extracts, 7, 298, 300, 315
- Fertilisation, 167, 339



- Fibroids of uterus, 340  
 "Flagellation," 175, 272  
     of malaria parasite, 323  
 Focusing, 33  
  
 Gametophytic tissue, 178  
 Globin, 322, 342  
 Granulation tissue, 338, 341  
 Granules, Altmann's, 52, *et seq.*, 223  
     counting, 273, 284  
  
 Hæmal glands, 180, 183  
 Hæmatin, 324, 331  
 Hæmoglobin, 128, 322, 397  
 Hæmolysins, 38, 98  
 "Hanging drop," 150, 419  
 Healing, 41, 168, 176, 319  
 Heat, 70, 78, 88  
 Hyaline cell, 4, 60  
 Hydrocyanic acid, 132, 148  
  
 Immunity, 9, 420  
 Incubator, 94, 98  
 Index of diffusion, 82, 94  
 Injury, 41  
 Irritation, 166, 170, 337, 348  
  
 Karyokinesis, 6  
 Katabolism, 361  
 Kinetic jelly, 133, 377, 407  
 Kreatin, 316  
  
 Lantern slides, 14  
 Leucocytes, Altmann's granules of,  
     256  
     bursting of, 107  
     divisions of, 252  
 Leukæmia, 175, 398  
 Life, climax of, 162  
 Lives of leucocytes, 132, 406  
 Lymphadenoma, 351  
 Lymphocytes in cancer, 393  
 Lymphocytes, mitosis of, 186  
  
 Maiotic divisions, 177, 239  
 Melanin, 322, 324  
 Malaria parasite, 323, 331  
 Malignant proliferation, 12  
 Methylene blue, 3  
 Metastasis, 165, 367  
 Mitosis, 185, 252  
 Morphine, 40, 118, 149  
 Moulds, 85  
 Mutations, 369  
  
 Neutral point, 89  
     red, 3  
 Nitro-benzol, 132, 148  
  
 Nuclei, lobes of, 5, 10, 43  
 Nuclein, 178  
  
 Opsonins, 130, 156  
 Osmosis, 59, 129  
 Osteo-arthritis, 163  
 Ova, 176  
 Oxygen, 148  
  
 Pancreas, 364  
 Phagocytosis, 155  
 Photomicrography, 3, 14, 17  
 Pigmentation, 322  
 Pilocarpine, 149  
 Plimmer's bodies, 181  
 Poisons, 8, 64  
 Potassium oxalate, 39  
 Precautions, 99  
 Protoplasm, 64, 69  
 Pseudopodia, 51, 56, 134  
 Putrefaction, 300, 350, 366  
 Pyridine, 149  
  
 Quinine, 149  
 Quinoline, 150  
  
 Red cells, 82, 117, 127, 294  
 "Red spots," 103  
 Reduction divisions, 239, 271  
 Reproduction divisions, 239, 271  
 Reproductive cells, 240  
 Resting-stage, 97  
 Rheumatism, 8  
  
 Salts, 37, 59, 86, 97  
 Sarcoma, 161, 397  
     cell-granules in, 291  
     melanotic, 322  
 Serum, 66, 374  
 Sexual form of malaria parasite, 323,  
     331  
 Sodium chloride, 38, 39, 78, 84, 88  
 Sodium citrate, 38, 44, 66, 76, 78, 88.  
 Somatic divisions, 239  
 Spermatozoon, 167, 339  
 Spindle, 12, 185, 257  
*Spirochata refringens*, 97  
 Staining, extent of, 75  
 Strychnine, 149  
 Suprarenal gland, 306, 345  
 Syphilis, 164, 364  
  
 Technique, for inducing mitosis, 245  
 Time, a factor of diffusion, 69, 78, 88  
 Tissue cells, 41  
 Transplantation of tumours, 369  
*Trepanema pallida*, 364

- Ulcers, 341, 345
  - malignant, 384
- Units, 76, 88, 97
- Unna's stain, 43, 52, 68, 77
- Urea, 99
- Vacuoles, diffusion, 104
  - ordinary, 103
- Variables, equation of, 145
- Vitality, 163, 363
- Wandering cells, 240
- Xanthin, 316
- X-ray cancer, 170











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